5-12-2019

The Biomechanics and Evolution of Air-Breathing in Anuran Tadpoles

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B.S., University of Connecticut, 2017

A Thesis

Submitted in Partial Fulfillment of the

Requirements for the Degree of

Master of Science

At the

University of Connecticut

2019

APPROVAL PAGE

Masters of Science Thesis

The Biomechanics and Evolution of Air-Breathing in Anuran Tadpoles

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2019

Acknowledgements

I would like to thank Dr. Kurt Schwenk, for without him, this thesis would surely have never been completed. Thank you, Kurt, for not only providing me with your ideas and thoughts on research, writing and presenting, but also teaching me how to think critically and instilling a strong desire for true scholarship in all my work going forward. We were not tadpole experts when we began this journey, and the incredibly rewarding journey towards becoming that has been the highlight of my degree. Thank you also to my committee members, Dr. Elizabeth Jockusch and Mark Urban, for your thoughtful and extremely useful comments on this thesis, as well as for all our conversations over these last several years. I also thank Dr. Paul Lewis for helping me wade through the field of phylogenetic trait reconstruction, your help was beyond instrumental in the creation of this second chapter. To my labmate, Amanda Hewes, thank you for being a friend, peer, resource and more this past year, especially over these few months. Your support has been extremely appreciated as I finish this process. I also thank members of my cohort: Genevieve, Laura, Sarah, Sumaira, Diler, Dipanjana and the rest for great times and all their support, both personal and academic. My project was partially funded by a 2018 Student Research Award, so thank you to the department and members of the committee that chose to award funding to my research. Finally, I thank my friends and family, who push me to be my best while also supporting all of my choices along the way, and to Emily Strack, for her patience and loving support over these past two years.

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Chapter 1

The Mechanics of Air-Breathing in Anuran Tadpoles.

Hyla versicolor Leconte, 1825 (Hylidae)

ABSTRACT

We describe the mechanics of air-breathing in the hylid tadpole *Hyla versicolor* Leconte 1825. Previous work has shown that the tadples of many species perform a breathing behavior that does not require breaking the water's surface tension to breathe (bubble-sucking). Tadpoles use this breathing behavior early in ontogeny and then switch to more typical breach-breathing later, when they are larger. We show here that *H. versicolor* tadpoles forgo breach-breathing entirely and perform bubble-sucking throughout ontogeny. Unlike other tadpoles, *H. versicolor* larvae perform the bubble-sucking behavior previously observed in other tadpoles (single bubble-sucking) as well as a novel, derived form of bubble-sucking we call 'double bubble-sucking'. There is a clear ontogenetic transition from single bubble-sucking to double bubble-sucking in *H. versicolor*, which is coincident with a morphological change in lung vascularization. A combination of functional, behavioral, and morphological evidence suggests that double bubble-sucking increases the efficiency of gas exchange by separating the deoxygenated air from the lungs from freshly breathed air to decrease mixing during breathing. We also comment on the potential significance of this finding for understanding the evolutionary history of vertebrate breathing mechanisms.

INTRODUCTION

The evolution of lungs was critically important for the origin of tetrapod vertebrates and the colonization of terrestrial environments by tetrapods. Lungs and air-breathing are often viewed as quintessentially terrestrial adaptations. Nevertheless, lungs evolved long before the water-land transition in the fish ancestors of tetrapods (Brainerd, 2015). A functional and evolutionary understanding of air breathing must therefore be sought within the context of obligate aquatic life. Anuran larvae may be useful models in this regard because, like ancestral fishes, they are fully aquatic organisms possessing both functional lungs and gills. Furthermore, tadpoles undergo a metamorphosis during which they transition into terrestrial adults, an ontogenetic process that mimics, if not actually recapitulates, the evolution of terrestriality.

The utility of this system as an evolutionary model is given additional weight by the recognition that anuran metamorphosis, and amphibian metamorphosis more generally, is likely to be a primary aspect of their biology rather than secondarily evolved (Hanken, 1999; Altig and McDiarmid, 1999). Tadpole fossils assignable to modern families are known from the Early Cretaceous (Gardner, 2016), true frogs from the Jurassic (Jenkins and Shubin, 1995), and stem-frogs from the Triassic (Rocek and Rage, 2000). Modern amphibians (Lissamphibia), including frogs, are likely to have evolved from a group of Devonian temnospondyls known as dissorophoids, which underwent metamorphosis from an aquatic larval form (e.g., Boy, 1974; Bolt, 1977; Schoch, 2002, 2014; Witzmann and Pfretzschner, 2003; Fröbisch et al., 2010; reviewed by Schoch, 2009). Although modern dipnoan fishes, the extant sister-group of tetrapods, may lack a larval stage, they are paedomorphic relative to their Mesozoic ancestors, which almost certainly possessed larvae and underwent metamorphosis (Joss and Johanson, 2007). Parsimony, therefore, suggests the unbroken historical continuity of an aquatic larval

stage throughout the history of modern amphibians (secondarily lost in some species). Thus, although modern frogs and their larvae are both morphologically derived, the use of lungs for breathing air in an aquatic larva and metamorphosis into a terrestrial, air-breathing adult form are likely to be retained, ancestral traits.

A consensus has not yet emerged regarding the evolutionary sequence that gave rise to lungs and air-breathing in vertebrates, largely owing to the lack of direct fossil evidence, which weakens our ability to infer the phylogenetic history of vertebrate lungs. Placoderm fishes offer some fossil evidence of lungs predating the origin of bony fish, but this evidence is not universally accepted. Devonian placoderms of the genus *Bothriolepis* have been suggested to possess paired, ventral sacs may be homologous to the lungs of modern dipnoan lungfish (Denison, 1947; Janvier et al., 2007; but see Perry, 2007; Goujet, 2011). Under the assumption of parsimony, the distribution of lungs in extant clades of vertebrates and their inferred presence in placoderms leads to the conclusion that lungs arose once at the base of the Gnathostomata (Liem, 1988).

Another approach to elucidating the history of air-breathing has been to examine the mechanics of breathing, i.e., 'ventilation'. Brainerd (1993, 1994) described two principal forms of ventilation in anamniote vertebrates—one using a two-stroke pumping mechanism and another using a four-stroke mechanism. In two-stroke breathing, buccal expansion draws air into the mouth or pharynx and compression forces it into the lungs. In contrast, four-stroke breathing requires two expansion-compression cycles in sequence. Four-stroke ventilation is characteristic of nearly all air-breathing actinopterygian fishes and a few, exceptional amphibians, whereas two-stroke ventilation is characteristic of all sarcopterygians, including lungfish and virtually all living amphibians. Brainerd (1994) suggests that the sarcopterygian two-stroke mechanism is

homologous to the ancestral gill irrigation pump mechanism found across all fishes, but that the actinopterygian four-stroke mechanism was derived from either suction feeding or 'coughing' mechanisms. On the basis of the different ventilation mechanisms, Brainerd (1994, 1995) argues that these breathing mechanisms arose independently in actinopterygian and sarcopterygian fish. Perry (2001), argues that if their breathing mechanisms arose independently, then perhaps this also implies separate origins of lungs in these two groups. This conclusion is also supported by some developmental evidence, which suggests that the putative placoderm lungs are not, in fact, homologous to other vertebrate lungs. Rather, it is suggested that vertebrate lungs arose independently from a shared ancestral "respiratory pharynx" (Wassnetzov, 1932; Perry et al., 2001; Perry and Sander, 2004).

Amphibians, generally, and tadpoles, specifically, may be particularly useful for reconstructing the evolutionary history of breathing in the context of ventilation mechanics. As alluded to above, several adult amphibian species are rare exceptions to the four-stroke/two-stroke, actinopterygian/sarcopterygian dichotomy. These include two salamanders (*Cryptobranchus* and *Amphiuma*) and two frogs (*Xenopus* and *Pipa* - both Pipidae), all of which exhibit four-stroke breathing mechanisms (*Amphiuma*: Brainerd et al., 1993; Martin and Hutchison, 1979, *Cryptobranchus*: E. L. Brainerd, *in lit*.; Simons et al., 2000, *Xenopus*: Brett and Shelton, 1979; Boutilier, 1984, *Pipa*: Fonseca et al., 2011). Notably, all are also permanently aquatic as adults. As far as is known, all other extant amphibians are two-stroke breathers, as expected under parsimony, although surprisingly few species and little diversity has actually been examined (*Rana*: DeJongh and Gans, 1969; Gans et al., 1969; Gnanamuthu, 1936; Vitalis and Shelton, 1990; West and Jones, 1974a; West and Jones, 1974b, *Bufo*: Jones, 1982; Macintyre and Toew, 1976; *Dermophis*: Carrier and Wake, 1995, *Siren*: Brainerd, 1998; Brainerd and

Monroy, 1998, *Necturus*: Brainerd et al., 1993; larval *Ambystoma*: Brainerd, 1998; adult *Ambystoma*: Simons et al., 2000). Missing from this assessment are larval anurans, which we believe have retained the ancestral, stem tetrapod traits of an aquatic habitat and (presumably) two-stroke air breathing. Until recently, however, almost nothing was known about the mechanics of tadpole air-breathing. In two previous papers we showed that lung development and air-breathing begin within a few days of hatching at minute body sizes in several species of tadpoles (two ranids, two hylids and *Xenopus*; Schwenk and Phillips, manuscripts a, b). Larger tadpoles accessed air to breathe by breaching the water's surface, as is typical of aquatic vertebrates. By contrast, small tadpoles were unable to break the water's surface tension and were forced to circumvent it by employing a novel breathing mechanism we call "bubble-sucking." During bubble-sucking, the surface of the water is sucked into the buccal cavity with a bubble of air, and while an open connection to the surface is maintained, the lungs expel air into the mouth. The connection to the air at the surface is then severed by mouth closure, forming a bubble within the buccal cavity that is then forced into the lungs by buccal compression. Whether the air is obtained directly via breaching or indirectly via bubble-sucking, we showed that tadpole breathing behavior uses a typical two-stroke mechanism characteristic of virtually all adult frogs and sarcopterygians, generally, and in this sense is not surprising.

However, the air-breathing behavior of the two hylid species we studied (*Hyla versicolor* and *Pseudacris crucifer*) departed from this pattern in two principle ways. a) Hylid tadpoles were never observed breach-breathing, as did ranid and *Xenopus* tadpoles. Rather, the two hylids appeared to bubble-suck throughout the larval period and well into metamorphosis. b) Hylid species also appeared to use more than one suction event per breath in some breathing bouts, something we never saw in other species. To better understand these differences, we examine

here the breathing mechanics of the hylid species *Hyla versicolor* (gray tree frog). We found that hylid frogs are the first vertebrates known to employ both two- and four-stroke breathing modes within a single individual over the course of its ontogeny. Given this unusual and unexpected breathing pattern in an aquatic frog larva and the association of four-stroke breathing mechanisms with the aquatic adults of some other amphibian species, hylids may offer insight into the relative lability of breathing mechanisms and the causal connections among environment, behavior, function, natural history and breathing efficiency.

MATERIALS AND METHODS

Animals

H. versicolor eggs were laid in small artificial ponds by wild individuals. The ponds consisted of either plastic five-gallon buckets or abandoned planters with shallow water and vegetation, located in an open field adjacent to woodland in Storrs, Connecticut, United States of America (41.788729, -72.221995). Eggs were transported back to the lab and raised in five-gallon glass aquaria. Tadpoles were not fed until they had consumed the egg mass and their remaining yolk, at which point they were provided with boiled lettuce ad libitum. Water was changed whenever it became turbid using either untreated well water or tap water that had been aged and aerated for at least two days (to avoid chlorine toxicity). The animal room was maintained on a 12-12 light-dark cycle.

Videography

We used an Edgertronic SC1, monochrome, high-speed video camera fitted with a Nikon 105 mm, f.2.8 macro lens to obtain slow-motion video of tadpoles air-breathing. For videography,

tadpoles were placed in a small, glass or plastic chamber that allowed unrestricted motion. A millimeter scale was placed within the field-of-view of the video frame against the back of the front pane of glass or plastic for later use during video analysis. Illumination was provided by three large banks of continuous (video) LED lights (500 LED lights, ikan®, Houston, TX), plus additional, supplemental lights as needed. High-speed videos were taken at frame rates of 300 to 1,000 frames per second (fps) (real-time video = 30 fps).

Dissection

Tadpoles were sacrificed by means of anesthetic overdose using a bath of 10% benzocaine ointment dissolved in water (AVMA, 2013). Tadpoles were then rinsed in deionized water and either dissected immediately under a dissecting microscope or fixed in ten percent formalin solution. Formalin-fixed specimens were later transferred to seventy percent ethanol for long-term preservation after at least one week in fixative. Dissections performed on fresh (unfixed) specimens allowed us to visualize the lungs while still inflated (most often deflated in fixed specimens).

Paraffin Histology

We prepared 19 individuals for standard paraffin histology. Specimens ranged from 3.3 mm to 12 mm in snout-vent length (SVL). Specimens were embedded in pure 100% paraffin and sectioned in the frontal plane at 6-10 µm to examine lung structure and vascularization. Sections were stained with either Harris' hematoxylin and eosin, or Weigert iron hematoxylin and picro-ponceau (Presnell et al., 1997). The latter stain provided better visualization of blood vessels for quantification of lung vascularization.

Video analysis

We used the freeware program Tracker® v.4.11.0 (Brown, 2017) to quantify the kinematics of air-breathing from high-speed videos. Using the measuring tool in Tracker® calibrated with a scale within the video frame, we measured tadpole snout-vent length (SVL) in mm. Each video was also scored by breathing mode (see below). We identified several discrete kinematic events that occur during a breathing cycle—attachment, bubble-suck I, lung empty, interval, bubble-suck II, compression, lung fill, and release). Designating 'attachment' as time zero, we calculated the time to initiation of each kinematic event and its duration using the frame counter tool in Tracker®. Duration in seconds was calculated by dividing the total number of frames by the frame rate (fps) at which the video was captured.

Histological analysis

We selected three individual sections per tadpole for analysis of lung vascularization. We standardized the selection of sections for each individual as much as possible: (a) we identified which lung (left or right) appeared to provide better quality sections and starting with the first, dorsal-most section in which lung tissue appeared, we counted all sections through the lungs, moving ventrally, until the initially (more-or-less) ovoid lung section became divided antero-posteriorly into two separate parts (this occurred because the lungs are tubular structures lying within the tadpole as dorsally curved arches). Any more ventral sections represented the anterior- and posterior-most ends of the lungs;, thus, we limited our analysis to the principal part of the lung represented by the set of the more dorsal sections; (b) we selected lung sections in three planes at 25%, 50%, and 75% of the total number of counted sections and used these to quantify vasculature in each individual. (c) If a section so-identified happened to be torn or otherwise

damaged, the nearest undamaged section was used instead. We quantified the degree of lung vascularization for each tadpole by examining each section under a microscope at 100-400X magnification and counting the number of blood vessels occurring along the lung's margins and septa. Blood vessel counts from the three sections were averaged to provide a single value for each individual.

Statistical analyses

We performed four different statistical analyses: (a) using the high-speed videos, we tested whether the duration of discrete kinematic events (e.g., suction, compression, interval, etc.) changed across ontogeny. This was accomplished by regressing the average duration of each kinematic phase against body length and using the F-statistics to assess potentially significant relationships. (b) We assessed whether the kinematics of the two observed breathing modes (single bubble-sucking and double bubble-sucking; see below) differed significantly in duration using two-sample t-tests. To do this, we identified five pairs of putatively homologous kinematic phases in both single- and double bubble-sucking for which duration time data could be obtained from the videos: "suction 1" (DBS) and "suction" (SBS), "suction 2" (DBS) and "suction" (SBS), "compression" (DBS) and "compression" (SBS) and "lung-fill" (DBS) and "lung-fill" (SBS). (SBS: single bubble-suck, DBS: double bubble-suck; see below). (c) We examined the temporal distribution of the two observed breathing modes (single bubble-sucking and double bubble-sucking) through the larval period using a combination of methods. First, we plotted the two breathing modes on the shared axis of length as a histogram in order to explore the data. We visually inspected the data for evidence of a behavioral shift in breathing mode with increasing size. Our inspection did indicate the presence of such a size-related shift, which we tested using

logistic regression to evaluate the probability of a tadpole using one breathing behavior over the other based on the continuous, dependent variable of body length (SVL), which was scaled and centered the continuous variable "length" prior to modeling. We anticipated that our model would reveal an ontogenetic transition from one breathing mode to another, which we identified as the body length at which the model predicted an equal probability of performing each behavior (pr[Double bubble-sucking] = 0.5). (d) We also examined the ontogeny of lung morphology using linear modeling. We regressed the average blood vessel count for each sectioned tadpole against its SVL. A preliminary examination of the data suggested the possibility of a sharp change in the number of blood vessels within the lungs that might best be modeled after splitting the data into two groups and modeling each group of points independently. We used the packages strucchange v.1.5-1 (Zeileis et al., 2002) and breakpoints v.1.5-1 (Zeileis et al., 2003) to find the body length most likely to represent a transition point from one model to another. This was accomplished with a breakpoints analysis that allowed our data to be split at any breakpoint, and then optimized that point to attain the highest log-likelihood of the model. After finding the best transition point and splitting the data into two groups based on the breakpoint, we fit each set of data points with its own linear model. We then compared the summed AIC scores of the two models for the split data to the AIC score of a single, linear model of the entire dataset to confirm that this split method was supported. All statistical analyses were performed in R v.3.4.3 (R Core Team, 2017).

Owing to practical issues related to maintaining and filming large numbers of tadpoles, we always filmed several to many individuals simultaneously, leading to a potential problem with pseudoreplication. It is theoretically possible, for example, that a few individuals did most of the breathing and our sample size is actually much smaller than we believe. To mitigate this

concern, we examined every video with great care, using individual identifiers such as size and coloration to rule out the possibility that multiple breaths were performed by the same individual. If the breathing individuals in different videos could not be positively assessed as different, then one of the videos was removed from the analysis. This process should greatly reduce any problem with pseudoreplication. Therefore, each data point is indicative of not only a breathing bout, but an individual. In those videos initially selected for statistical analysis we rarely (<10) observed the same individual perform both breathing modes. To avoid any bias by assigning a single mode to such a tadpole, we excluded these individuals from all statistical analyses.

RESULTS

Breathing modes

As noted by Schwenk and Phillips (manuscript a), *Hyla versicolor* tadpoles develop lungs, inflate them and begin air-breathing just a few days after hatching at remarkably small body sizes (3 mm SVL). All six air-breathing tadpole species we have so far examined are capable of breathing air without breaching the water's surface by employing a novel behavior we recently call "bubble-sucking" (Schwenk and Phillips, manuscripts a and b). All of these species employ bubble-sucking at small body sizes early in the larval period before transitioning to breach-breathing, where the tadpole does break the surface tension to gain access to gaseous air. We have argued that bubble-sucking is an adaptation to circumvent the mechanical constraint of surface tension, because very small the tadpoles are unable to break the water's surface (breach) to access air (Schwenk and Phillips, manuscript a).

H. versicolor (and the other hylid for which we have preliminary data, *Pseudacris crucifer*) differs from other species in two important ways. First, even as they grew large and fast

enough to break through the water's surface tension, they did not transition to 'breach breathing'. Intead, they continued to bubble-suck throughout the larval period, well into metamorphic climax. During occasional, explosive bouts of swimming, *H. versicolor* tadpoles easily broke through the water's surface, but they never breathed while having access to the air. Second, *H. versicolor* (and *P. crucifer*) tadpoles exhibited two distinct types of bubble-sucking: 'single bubble sucks' (SBS) and 'double bubble sucks' (DBS) (Table 1.1). Double bubble-sucks are similar to single bubble-sucks except that a second suction event occurs immediately following the first (see below). In addition, two individuals were occasionally observed to use 'triple bubble-sucks'; however, these were likely aberrant as the two individuals had been raised separately in a five-gallon bucket in which they grew unusually large and did not show any signs of metamorphosing (sacrificed at 15.5 and 16.5 mm SVL, Gosner stages 26-30). Furthermore, SBS and DBS together constituted more than 98% of the total observed breathing bouts. Therefore, we do not consider triple bubble-sucks further, focusing on the mechanics of single- and double bubble-sucking, which are described below.

Although we observed DBS much more often than SBS in our videos (Table 1.1), we caution that the significance of the relative frequencies of SBS and DBS in our data is unclear. The difference may be an artifact of observation bias due to the relative ease of data collection for larger individuals, it may reflect more frequent breathing in larger individuals, or it may result from the fact that more total time was spent by tadpoles in later developmental stages when DBS is more frequent. We believe that the latter possibility is an important factor because tadpoles exhibited an ontogenetic shift from single- to double bubble-sucking relatively early in the larval stage (see below).

The mechanics of single and double bubble-sucking

Single bubble-sucking

As noted, SBS in *H. versicolor* is similar to bubble-sucking behavior we described previously for other tadpoles (Schwenk and Phillips, manuscript a), and in the most detail for green frog tadpoles (*Rana clamitans*) (Schwenk and Phillips, manuscript b*)*. We observed SBS throughout tadpole ontogeny, but at much greater frequency in smaller (younger) individuals (see below). In fact, the smallest breathing tadpole for which we observed air-breathing performed SBS at only 3.08 mm SVL (Gosner stage 25). The largest SBS individual we recorded was 10.3 mm SVL (Gosner stage 26-30), but this individual was also recorded performing DBS, so was not included in the analysis (see above).

SBS is initiated when a tadpole swims upward and attaches its mouthparts to the underside of the water's surface. Attachment is a key difference between bubble-sucking and breach breathing, and marks the beginning of a bubble-sucking breathing cycle. Leading up to attachment, the tadpole typically rocks from side-to-side with its mouth closed, pushing upward against the undersurface of the water. The tadpole then opens its mouth fully, forming its oral disc into a circular cup that is pressed to the surface where it adheres. Owing to the subterminal position of the mouth in *H. versicolor*, the tadpole often orients its body obliquely with its ventral side up so that the mouthparts are aligned with the water's surface (Fig. 1.1A). Immediately following attachment, buccal expansion draws the water's surface layer into the mouth, forming an air bubble within the buccal cavity (Fig. 1.1B). While the bubble remains connected to the air above the surface via a narrow stalk, the lungs empty into the bubble. The tadpole then closes its mouth, severing the bubble's connection to the atmosphere ("pinch-off") (Fig. 1.1C). Following pinching-off and a short pause, the tadpole elevates the buccal and

pharyngeal floors, compressing the air-bubble and forcing air into the lungs (Fig. 1.1D). Air remaining within the buccal cavity after the lungs fill is expelled when the tadpole opens its mouth and evevates the buccal floor, usually as the tadpole swims away from the surface (Fig. 1.1E). Figure 2A provides a simplified schematic of the kinematic events described above.

Note that we did not directly observe lung emptying during any recorded single bubble-sucks in *H. versicolor*, but infer that it occurred for several reasons. First, lung emptying and to a lesser extent, lung filling is difficult to observe in the very small *H. versicolor* tadpoles that exhibit SBS behavior. Visualization requires external bulging of the lungs, which often occurs in larger tadpoles, but is rarely evident in small individuals. It also requires that the tadpole be oriented in dorsal view at high magnification and precisely in focus, which only occurred in a small fraction of our videos. Regardless, lung filling is impossible if the lungs have not previously been emptied and *H. versicolor* tadpoles were never observed to expel air bubbles at any time other than the end of a breathing cycle. We also confirmed that small tadpoles that only performed SBS did have inflated lungs with dissections of unfixed individuals. Finally, as noted previously, single bubble-sucking behavior in *H. versicolor* is virtually identical to bubble-sucking behavior observed in *Rana* and *Xenopus* tadpoles, and lung emptying in these species occurs exactly as described above.

The average duration of a SBS bout is 0.444 ± .13 seconds (Table 1.2; Fig. 1.2B). We did not find any relationship between SVL and the duration of either the total breathing event or any individual phase of a breathing event, [Supp. Fig. 1.1: total duration vs. length: (ß = -0.012, p = 0.408); suction vs. length: (ß = 0.00002, p = 0.99); compression vs. length: (ß = -0.012, p = 0.338); lung-fill vs. length: (ß = 0.0002; p = 0.946)].

Double bubble-sucking

DBS is the most frequent form of breathing we observed in *H. versicolor* (Table 1.1). It was exhibited by tadpoles as small as 5.1 mm SVL (Gosner stage 25) up through metamorphic climax. DBS persisted until froglets climbed up and out of the water, at which point they began to perform typical, aerial frog breathing.

DBS is distinguished from SBS by the presence of two suction events rather than one. Otherwise, the breathing modes are similar, sharing many kinematic phases (Fig. 1.2). Nevertheless, several differences are notable. First, direct visualization of lung emptying was often possible during suction I in the larger tadpoles exhibiting DBS. Often the lungs were directly observable externally because when filled with air they bulged slightly, or the distal tip of the lung was visible through a small, unpigmented patch of skin in which air could be seen moving into or out of the lung. Lung-emptying was observed to be explosive, occurring in just a few milliseconds. In cases in which lung emptying could not be seen directly, a whole-body twitch often marked the event. These observations confirmed that lung emptying occurs during the first bubble-suck while the bubble remains attached and open to the surface (Fig. 1.1G). Second, pinch-off does not occur at the end of the first suction phase. Rather, immediately following lung emptying, the buccal bubble snaps back to the surface, presumably because its surface tension is elastically stretched when sucked into the mouth. This may occur because buccal musculature is relaxed or because the mouth is opened further. Following suction I, a very short interval phase occurs while the tadpole remains attached to the water's surface by its oral disk (Fig. 1.1H). This is followed by a second bubble-suck event (suction II) that is identical to the single suck described above, terminating with pinch-off and followed by a compression phase, lung filling and the release of excess air (Fig. 1.1I-M; Fig. 1.2C).

Average total DBS duration was .518 ± .08 seconds (Table 1.2; Fig. 1.2D). We found significant relationships between the duration of kinematic phases and body length in some but not all breathing phases. DBS total duration was positively correlated with body length (ß = 0.014, p = 0.037), as were suction 1, (ß = 0.0025, p = 0.028) and suction II (ß = 0.0043, p = 0.00037) (Supp. Fig. 1.2). The phases lung-empty (ß = 0.0004, p = 0.467), lung-fill (ß = -0.0013, p = 0.613) and interval (ß = -0.0001, p = 0.7757) did not significantly differ over length.

Kinematic differences between single and double bubble-sucking

We found that while the phases of breathing were qualitatively similar between SBS and DBS, the timing of some events differed significantly between the two modes in some but not all pairwise comparisons (Table 1.2; Suppl. Fig. 1.3). The total duration of DBS was significantly longer than that of SBS [Total Duration$_{DBS}$ = 0.518 s; Total Duration$_{SBS}$ = 0.445 s; p = 0.0186] and suction I of DBS was longer than the suction phase of SBS,[($S1_{DBS}$) = 0.112 s; (S_{SBS}) = .0704 s; p = 2.63E-12], while Suction II of DBS did not differ from SBS suction [($S2_{DBS}$) = 0.0669 s; (S_{SBS}) = 0.0704 s; p = 0.456]. Compression did not differ significantly between the two modes[(C_{DBS}) = 0.333 s; (C_{SBS}) = 0.374 s; p = 0.136]. We did find a highly significant difference between the duration of lung-fill in DBS and SBS [(LF_{DBS}) = 0.0269 s; (LF_{SBS}) = 0.0838 s; p = 6.33E-07], with the average DBS lung-fill being several times faster (shorter) than that in SBS.

Ontogenetic transition from single to double bubble-sucking

The frequency distribution of single bubble-sucks and double bubble-sucks according to SVL shows that breathing mode is not randomly distributed through the larval period (Fig. 1.3A). Rather, it suggests a developmental transition from SBS behavior to DBS behavior such that

very small tadpoles only single bubble-suck and very large tadpoles only double bubble-suck, with an intermediate size range during which single sucking occurs at low frequency. Indeed, our logistic regression indicates that the relationship between SVL and breathing mode was highly significant ($p = 2.65e-09$). The sigmoid curve given by the logistic regression exhibits an inflection point at approximately 6 mm (Fig. 1.3B). We defined the transition point in our model as the SVL at which the model predicts an equal probability of performing either breathing mode, which was estimated to be 5.70 mm SVL. Finally, we explored the possibility that this transition in breathing mode was associated with vascularization of the lungs. Using a breakpoint analysis on the lung vasculature data, we found that the body length at which a transition from one model to another was most likely to occur was at 6 mm. We therefore split our data into two sets—those points with body lengths $\geq$ 6 mm, and those $<$ 6 mm. We found that the best fitting linear models for the two data sets had equal, positive slopes (Fig. 1.3C). To confirm that the split data model was appropriate, we compared its total AIC score to a single linear model of the total data set. The summed AIC scores of the split models is 59.55 and the AIC of the single model is 65.69, which supports the split model's use. We conclude that at approximately 6 mm SVL, the lungs undergo a rapid maturation event during which they greatly increase the number of blood vessels (1-4 vs. 5-15 vessels per section; Fig. 1.4) and by inference, the extent to which the lungs are able to serve as a sites of gas exchange. Furthermore, this rapid increase in lung vascularization is tightly correlated with a shift in breathing mode, from single bubble-sucks to double bubble-sucks.

DISCUSSION

Bubble-sucking specialization in *Hyla versicolor*

Our previous work has shown that when tadpoles are too small or slow to break the water's surface tension they bubble-suck, a behavior that circumvents the need to breach the surface to access air for breathing (Schwenk and Phillips, manuscript a). When large enough, tadpoles switch from bubble-sucking to typical breach-breathing. However, we show here that *H. versicolor* tadpoles never make this transition, despite growing large enough to easily breach the water's surface. *H. versicolor* tadpoles grow to body sizes comparable to species that breach (attaining body lengths of 16+ mm at metamorphosis), and yet they continue bubble-sucking behavior into metamorphic climax (Fig. 1.2A). These observations suggest that the failure of *H. versicolor* to breach-breathe is not a consequence of a physical constraint (Schwenk and Phillips, manuscript a), but rather the consequence of an adaptive specialization such that breach-breathing is replaced by an alternative breathing mode.

Morphological features of *H. versicolor* also indicate potential bubble-sucking specialization. Compared to bubble-sucking amphibian larvae that transition to breach-breathing (e.g. *Rana sylvativa*, *R. clamitans*, *Xenopus sp.*, and the larvae of the salamander *Ambystoma maculatum*), *H. versicolor* tadpoles have exceptionally large oral disks relative to their body size (personal observation). These large mouthparts may enhance their ability to attach to the underside of the water's surface by increasing the contact area with the surface layer, thereby enhancing their ability to bubble-suck. Some species of tadpoles that specialize as neustonic surface-feeders have similarly large mouthparts that function in surface attachment during feeding (e.g. *Megophrys*, *Phasmahyla*, etc. Inger, 1985; Wells, 2007). We note in this regard that *H. versicolor* tadpoles are prodigious surface feeders, as well (unpublished observations). The

same underlying principles apply to both surface-feeding and bubble-sucking, as both require a secure attachment to the surface.

Perhaps the most critical evidence in support of the specialization hypothesis is our discovery of double bubble-sucking. Although our taxon sampling remains small, the phylogenetic distribution of single vs. double bubble-sucking species (Fig. 1.5) implies that exclusive SBS is the ancestral breathing mode for frog larvae, and that the addition of DBS is a derived condition in *H. versicolor*, and possibly all of Hylidae, as we have also observed DBS in *Pseudacris crucifer*, another hylid frog (unpublished data). Finally, we suggest that DBS is a more efficient breathing mode than SBS due to its separation of excurrent and incurrent air-streams, which prevents mixing of fresh and depleted air (discussed further below). This notion is supported by the finding that a rapid increase in lung vascularization (and therefore, gas exchange efficiency) is tightly correlated with the transition in breathing mode. Thus, the evolutionary innovation of DBS may be the result of bubble-sucking specialization in hylid frogs to increase respiratory efficiency as tadpoles. The very brief time a breaching tadpole remains above the surface would seem to preclude the ability to perform two, sequential suction events. The average duration of the suction event in single bubble-sucking is less than half that of suction I and suction II combined (Fig. 1.2). A firm attachment to the surface (the key element of bubble-sucking) allows *H. versicolor* to perform this more complex behavior without any apparent time constraint. In combination, the evidence presented here uniformly points to adaptive specialization in *H. versicolor* (and other hylids) for bubble-sucking and respiratory efficiency.

Ontogenetic transition of breathing mode

An ontogenetic change from SBS to DBS in *H. versicolor* represents a transition from the ancestral to a derived breathing mode. Under what circumstances did this new breathing mode arise and why would a novel breathing mode have evolved in a species without entirely replacing the ancestral mode? We show that SBS is the more prevalent breathing mode before tadpoles attain a body length of 5.7 mm, whereas DBS is more prevalent at larger sizes (Fig. 1.3). This ontogenetic transition in breathing behavior was found to correspond closely with a similar transition in lung development, from nearly avascular to vascularized. This concordant shift in breathing mode and lung morphology is unlikely to be a coincidence. It suggests that SBS behavior is largely non-respiratory, and that DBS is the primary respiratory air-breathing mode in *H. versicolor*. This is further supported by a functional comparison of SBS and DBS. Single bubble-sucks have no apparent way to prevent mixing of deoxygenated air from the lungs with freshly breathed air from the atmosphere (Fig. 1.2). Double bubble-sucks however, use two separate suction events, the first to empty the lungs and the second to supply fresh air for lung-filling. Therefore, given the potential increase in efficiency for gas exchange, a transition to DBS behavior is only sensible if it co-occurs with lung vascularization.

If SBS is non-respiratory, then why do it at all? One possibility is that inflated lungs have utility for functions other than respiration. Gee and Waldick (1995), for example, showed that *H. versicolor* tadpoles use inflated lungs to promote neutral buoyancy, which may help reduce the energetic cost of swimming and holding position in the water column. Alternatively, filling the lungs with air early in ontogeny might be necessary for proper lung development. Pronych and Wassersug (1994) found that when *X. laevis* tadpoles were denied access to air after hatching, their ability to develop and inflate lungs was delayed and the probability of successful

metamorphosis significantly decreased. Buoyancy and developmental necessity are not mutually exclusive hypotheses however, as both could provide selective pressure for tadpoles to inflate their lungs prior to any role in gas exchange. Hatchling *H. versicolor* tadpoles are very small, possess functional gills, permeable skin, and presumably have relatively low oxygen demands. Tadpoles perform SBS at this pre-respiratory stage because, presumably DBS takes longer (Fig. 1.2), uses more energy, and increases the likelihood of predation by increasing time spent at the surface (Baird, 1983; Branch, 1983; Feder, 1983). Regardless, DBS would confer no benefit while the lungs remain avascular. It is therefore reasonable to infer that any costs associated with DBS would maintain SBS behavior in small tadpoles.

In summary, we suggest that for hatchling *H. versicolor* tadpoles, the combination of gills and cutaneous respiration is adequate for their gas exchange needs. Within days of hatching, tadpoles begin to inflate their simple, avascular lungs for hydrostatic and/or developmental purposes. There is no benefit to performing DBS at this stage, as it requires more time and presumably, more energy, than SBS. As the tadpoles grow, their surface area to volume ratio decreases while their energy demands increase. At some point in growth, presumably around 6 mm body length, branchial and cutaneous respiration become insufficient and an additional site of gas exchange becomes necessary. At this point, tadpoles develop the requisite lung vasculature and initiate DBS to increase the efficiency of breathing.

Tadpole breathing in the context of respiratory pumps

As noted in the Introduction, actinopterygian fish typically perform four-stroke breathing, while sarcopterygians perform two-stroke breathing. There are several exceptions to this pattern, however, including several aquatic amphibians that perform four-stroke breathing. The only

tadpole with its air-breathing mechanism previously described in detail, *R. clamitans*, performs two-stroke breathing throughout its ontogeny while transitioning from bubble-sucking to breach-breathing (Schwenk and Phillips, manuscript b). We show here that *H. versicolor* tadpoles transition from single bubble-sucks, a two-stroke air-breathing mechanism, to double bubble-sucks, a four-stroke air-breathing mechanism.

The terms "two-stroke" and "four-stroke", coined by Brainerd (1993) in reference to two-stroke and four-stroke piston engines, refer to the number of distinct, bucco-pharyngeal movements that occur during a single breathing bout (emptying and filling of the lungs). Two-stroke breathers use a single pharyngeal expansion to fill the pharynx with freshly breathed air and at the same time also empty the lungs into the buccal cavity, such that the two airstreams share an airspace. They then close the mouth (or nares) and compress the air-filled bucco-pharyngeal cavity to fill the lungs (Brainerd et al., 1993) (Fig. 1.6). These events closely mirror the kinematic stages of SBS. Four-stroke breathers use four distinct movements to ventilate the lungs. They expand the buccopharyngeal space to empty the lungs, then contract it to expel the depleted air to the atmosphere. They then expand the buccopharyngeal space a second time to suck in fresh air. Finally, they close the mouth or nares and compress the fresh air into lungs (Brainerd et al., 1993). This description matches our observations of DBS, with one difference: *H. versicolor* tadpoles empty the lungs during suction I when fresh air is drawn into the mouth for the first time. This mixed air is then expelled.

The functional significance of initially drawing in air before lung-empty during the first suction event is unclear, as it should be possible for tadpoles to empty the lungs while submerged, releasing a bubble of expelled air into the water. Indeed, this is what actinopterygian fish do; i.e, they empty the lungs into the pharynx and expel air from the lungs as they rise to the

surface (Brainerd et al., 1993) (Fig. 1.6). We believe that this difference reflects the fact that DBS in *H. versicolor* is derived from SBS, a two-stroke breathing mode. It is also the case that in those aquatic salamanders in which four-stroke breathing is also secondarily derived two-stroke breathing, that a breathing bout is initiated by an initial suck of fresh air that is breathed out with the air from the lungs (Martin and Hutchison, 1979; Brainerd et al., 1993; Simons et al., 2000; Brainerd, *in lit.*) (Fig. 1.6). In all such secondarily evolved examples of four-stroke mechanisms, breathing is initiated with a suction event that is apparently functionally unnecessary for emptying the lungs. As such, an initial suction event may represent a vestige of the evolutionary transformation from two-stroke to four-stroke breathing. The lack of such an initial suction event in actinopterygian fish supports the hypothesis of independent evolutions of air-breathing in actinopterygian fish and sarcopterygians as suggested by Brainerd (1994) and Perry et al. (2001).

The kinematic differences between two-stroke and four-stroke breathing are often viewed through the lens of efficiency. Because four-stroke breathing clearly prevents mixing of oxygenated and deoxygenated air, while two-stroke breathing does not, some authors have argued that two-stroke breathing is a comparatively inefficient respiratory mode (Bishop and Foxen, 1968). Others have challenged this idea, suggesting that adult frogs, (two-stroke breathers), circumvent this problem by forming a "jet-stream", sending the excurrent airflow from the lungs along the roof of the mouth to avoid mixing with the fresh, incurrent air (deJongh and Gans, 1969; Gans et al., 1969). These findings could not be replicated, however (Vitalis and Shelton, 1990), and should be viewed with some skepticism. Nevertheless, Brainerd (1998) noted some differences among aquatic salamanders, finding that two-stroke breathing, larval *Ambystoma tigrinum* are able to limit mixing to 20% of the air compressed into the lungs owing to the difference between lung and pharyngeal volume. On the other hand, *Amphiuma*

tridactylium (a four-stroke breather) is able to reduce mixing to 0%. If this difference is functionally significant, then aquatic organisms may be more affected than terrestrial organisms and therefore more likely to evolve four-stroke breathing, as they are subject to selection limiting the frequency of air-breathing owing to increased energetic costs associated with rising to the surface or increased exposure to predation. Terrestrial organisms face no such costs because they can breathe in constant, cyclic bouts, which mitigates any effects of mixing (e.g. Carrier and Wake, 1995; Brainerd, 1999).

As the first known example of a species that performs both two-stroke and four-stroke breathing modes within an individual's lifetime, *H. versicolor* might provide new insight into the similarities and differences between the two breathing modes. Previously, comparisons between the two forms of breathing could only be made across species, introducing multiple confounding effects, including phylogeny. *H. versicolor* is now the best-known system to study the mechanical and physiological differences between two-stroke and four-stroke breathing. However, at least one other hylid (*P. crucifer*) and possibly other frog species may also perform both breathing mechanisms over the course of their development. Brett and Sheldon (1979), for example, suggested that *Xenopus laevis* adults breathe using a four-stroke pump. Since our initial observations of *Xenopus* tadpoles show that they employ two-stroke breathing (Schwenk and Phillips, manuscript a), this may represent another example of an ontogenetic transition in breathing mechanism among frogs. It is noteworthy in this context that, unlike the vast majority of frogs, *Xenopus* adults are fully aquatic and may face many of the same costs associated with two-stroke breathing in aquatic salamanders, suggesting again increased selection for more efficient breathing.

Amphibians provide a unique opportunity to study breathing patterns because species and individuals often span a large range of ecological space and are subject to diverse and often disparate environmental pressures over the course of ontogeny. Frogs, in particular, undergo a complete metamorphosis, such that their aquatic larvae inhabit a completely different environment than the terrestrial adults. This radical environmental change must certainly affect the nature of selection acting on the respiratory system. We noted above, for example, that terrestrial organisms may have lower costs for two-stroke breathing, as their environment allows them to continuously respire, whereas the costs associated with air-breathing in an aquatic environment are likely to severely limit time spent at the surface, precluding the possibility of continuous breathing (Feder, 1983). Like most frogs, *H. versicolor* occupies a considerable range of ecological space over the course of its ontogeny. As aquatic larvae, *H. versicolor* tadpoles perform two-stroke breathing early in the larval period and then switch to four-stroke breathing, presumably to increase the efficiency of air-breathing. Following metamorphosis, *H. versicolor* tadpoles spend the remainder of their lives as terrestrial/arboreal tree frogs. We might predict that *H. versicolor* adults would therefore have no need to continue four-stroke breathing as adults, and indeed, this is the case. Upon completing metamorphosis, *H. versicolor* adults, like other typical adult frogs (as far as is known), perform typical two-stroke breathing as described by Gans et al. (1969) (unpublished data). Thus *H. versicolor* individuals change breathing modes twice over the course of their lives, from two-stroke to four-stroke and back to two-stroke! This unprecedented series of transitions may be indicative of the evolutionary lability of the pump mechanisms underlying breathing modes. Not enough anuran larvae have been examined in this context; it is possible that some other amphibian larvae have evolved four-stroke breathing if

they have experienced selection for increased respiratory efficiency of the lungs during their aquatic phase.

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TABLES

Table 1.1 Frequency of different breathing modes in *Hyla versicolor* tadpoles.

BREATHING MODE	INDIVIDUALS (N)	PERCENT TOTAL
Single Bubble-suck	27	17.5
Double Bubble-suck	125	81.2
Triple Bubble-suck	2	1.3
Breach-breath	0	0.0

Table 1.2 Timing of kinematic phases of different breathing modes.

Single Bubble-sucking		Double Bubble-sucking	
KINEMATIC PHASE	**Mean Duration (s)**	**KINEMATIC PHASE**	**Mean Duration (s)**
Suction	0.07	Suction I	0.112
		Suction II	0.067
Compression	0.374	Compression	0.333
Lung-Fill	0.084	Lung-Fill	0.027
Total Breath	0.445	Total Breath	0.518

FIGURES

Figure 1.1 Kinematic Phases of Single and Double bubble-sucking. **A-E:** single bubble-sucking; **A:** Attachment; **B:** Suction; **C:** Pinch-off; **D:** Compression; **E:** Release; **F-L:** double bubble-sucking; **F:** Attachment; **G:** Suction I; **H:** Interval; **I:** Suction II; **J:** Pinch-off and first part of compression; **K:** Second part of compression (dashed circle highlights the now inflated tip of the lung, which emerges at the tadpole's posterior); **L:** Release.

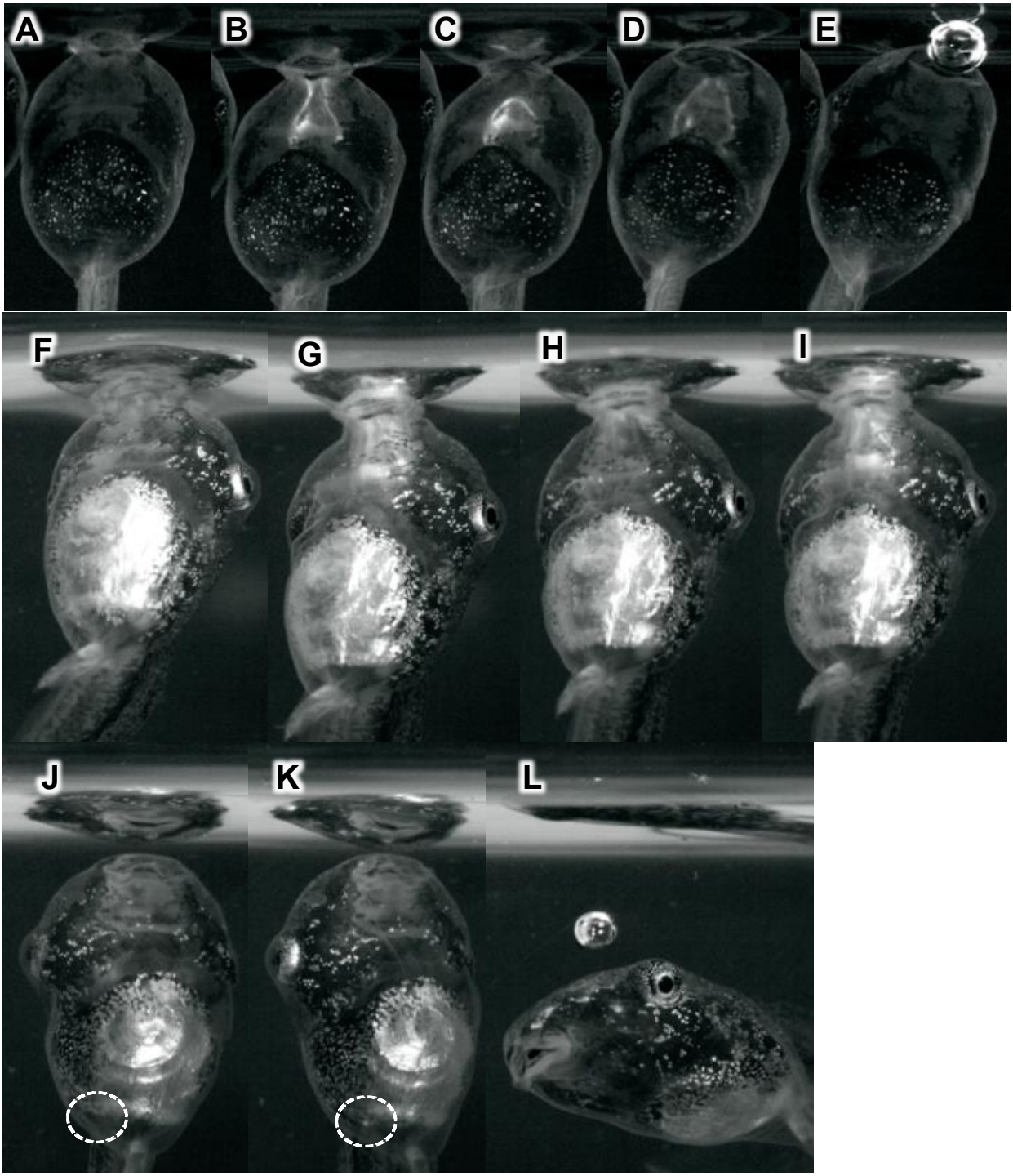

A
B
C
D
E
F
G
H
I
J
K
L

Figure 1.2: Simplified schematic of air breathing in *Hyla versicolor*. **A**: The kinematic phases of single bubble-sucking. The five small figures depict attachment, suction (and lung empty), pinch-off, compression and release. The colored bars below each schematic refer to B. **B:** Timing of the kinematic events of single bubble-sucking. The length of each colored section represents the mean duration of each kinematic phase. Note that because we could not visualize lung-emptying behavior in single bubble-suck videos, therefore the exact timing of this phase is unknown. The dotted blue lines indicate approximately where we infer lung empty occurs (see text). **C:** The kinematic phases of double bubble-sucking as indicated in (D). The colored bars below each figure refer to part D. Note that deoxygenated air is colored red, oxygenated air is colored blue and mixed air is colored maroon. **D:** Timing of the kinematic events of double bubble-sucking shown at the same scale as (B). The length of each colored section represents the mean duration of each kinematic phase. **E:** Between breathing bouts tadpoles swim fully submerged with lungs (Lu) full of deoxygenated air and the buccal cavity (Bu) empty or filled with water. In A and C, deoxygenated air is colored red, oxygenated air is colored blue and mixed air is colored maroon.

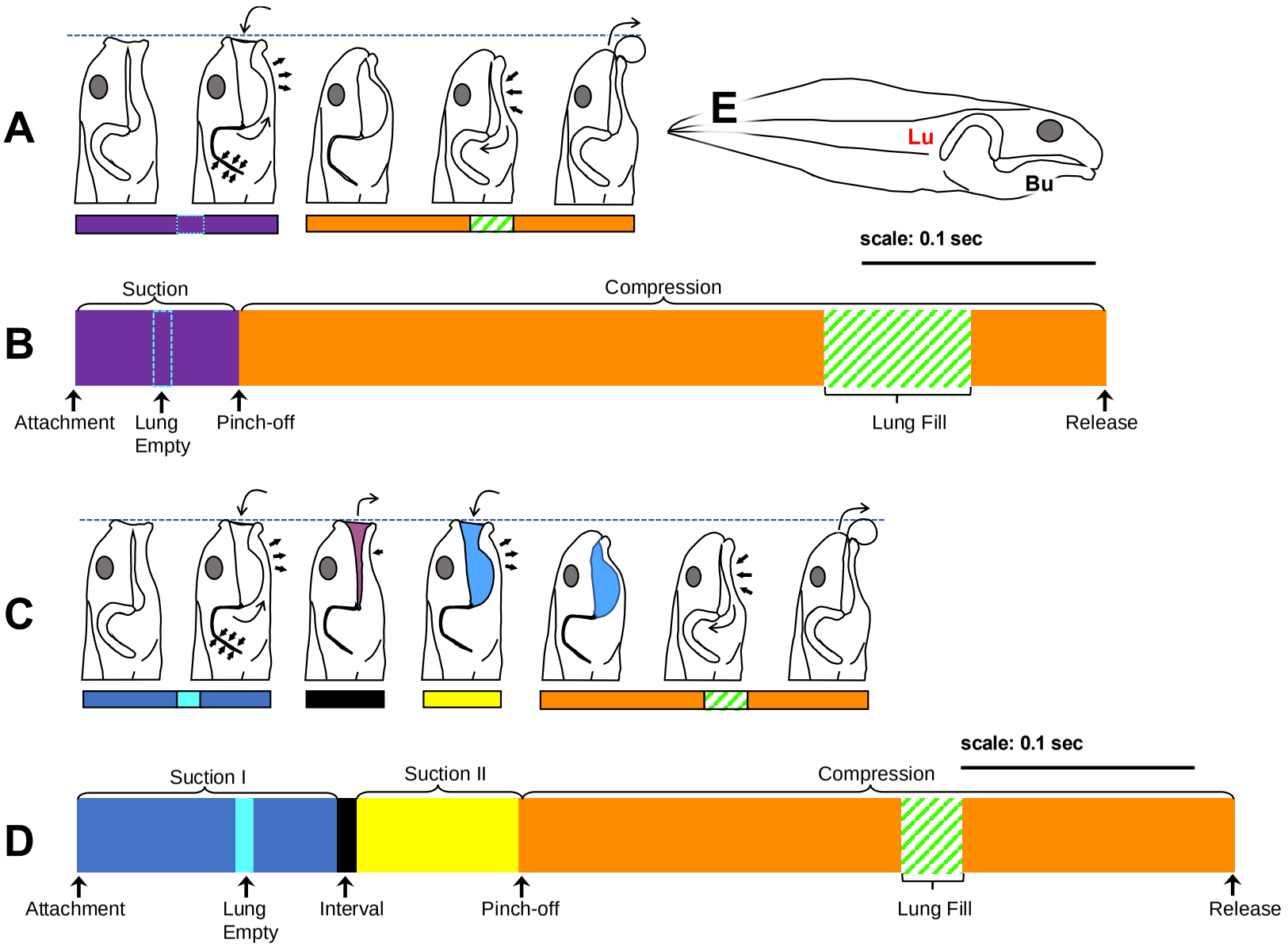

A
E
Lu
Bu
scale: 0.1 sec
B
Suction
Compression
Attachment
Lung Empty
Pinch-off
Lung Fill
Release
C
scale: 0.1 sec
D
Suction I
Suction II
Compression
Attachment
Lung Empty
Interval
Pinch-off
Lung Fill
Release

Figure 1.3: Changes in breathing behavior and lung morphology over ontogeny. **A:** Histogram showing the number of recorded instances of single bubble-sucking (red) and double bubble-sucking (blue). **B:** The probability of performing a double bubble-suck through ontogeny. The dotted line indicates the SVL at which the probability of performing a double suck or a single suck is equal (50%). **C:** Regressions of pulmonary blood vessel number vs. body length using a split dataset (see text). Red dots indicate single bubble-sucks and blue dots indicate double bubble-sucks. The transition from low vascularization to high vascularization was independently calculated to occur at 6 mm SVL.

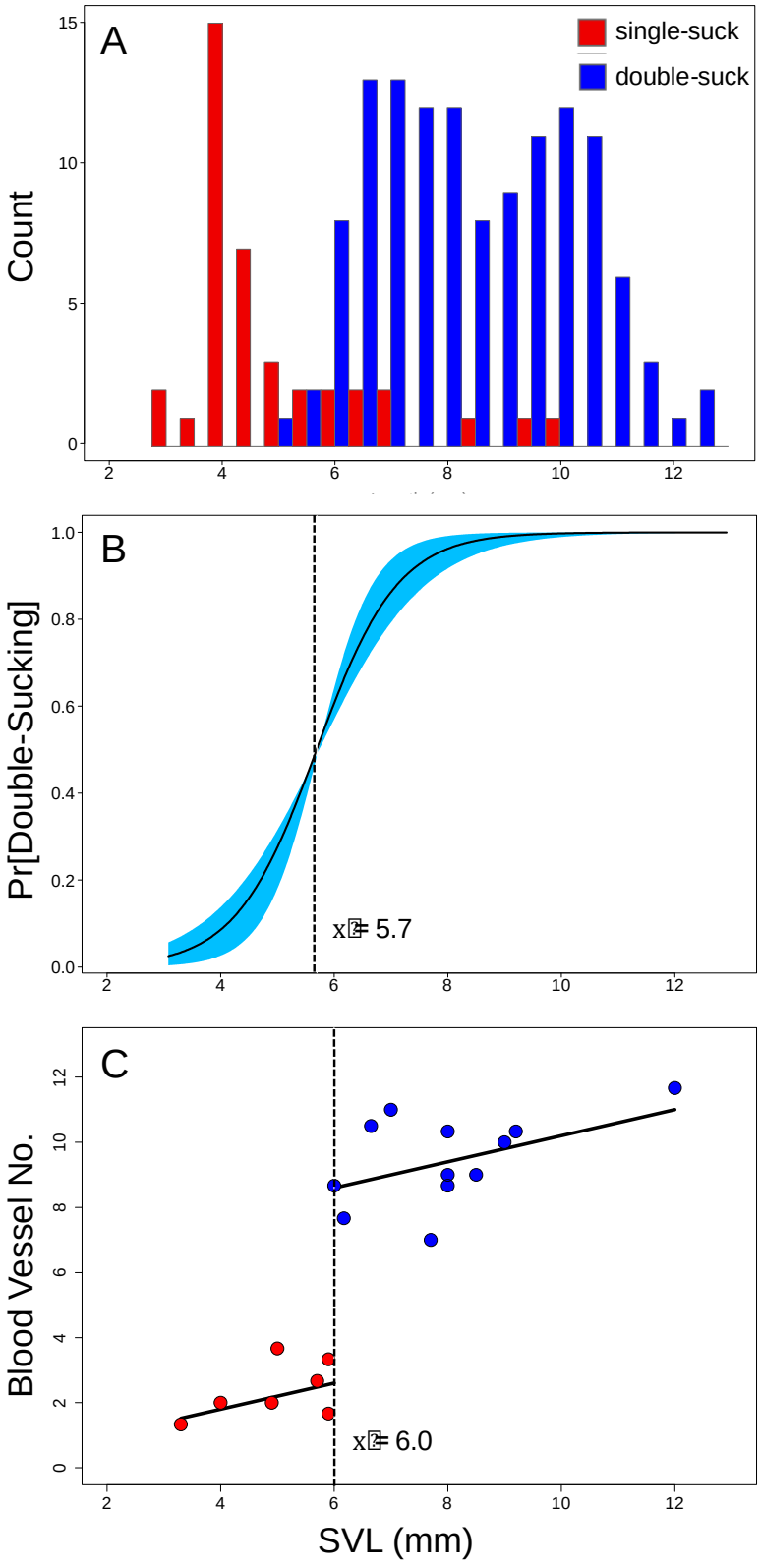

A
single-suck
double-suck
Count
15
10
5
0
2
4
6
8
10
12
B
Pr[Double-Sucking]
1.0
0.8
0.6
0.4
0.2
0.0
x = 5.7
C
Blood Vessel No.
12
10
8
6
4
2
0
x = 6.0
SVL (mm)

Figure 1.4 : Figure 4 : Frontal sections through *Hyla versicolor* lungs. **A:** A pre-transition tadpole (5.9 mm SVL). Note the general lack of structure and obvious vasculature. **B:** A post-transition tadpole (9.2 mm SVL) showing incipient formation of septa with associated blood vessels.

anterior
blood vessel
B
A

Figure 1.5 : Evolutionary relationships of single and double bubble-sucking in larval amphibians. The two hylid frogs (*Hyla* and *Pseudacris*) are the only taxa known to perform double bubble-sucking (indicated in red); all other species perform only single bubble-sucking (black). Evolutionary relationships from Pyron and Wiens (2013).

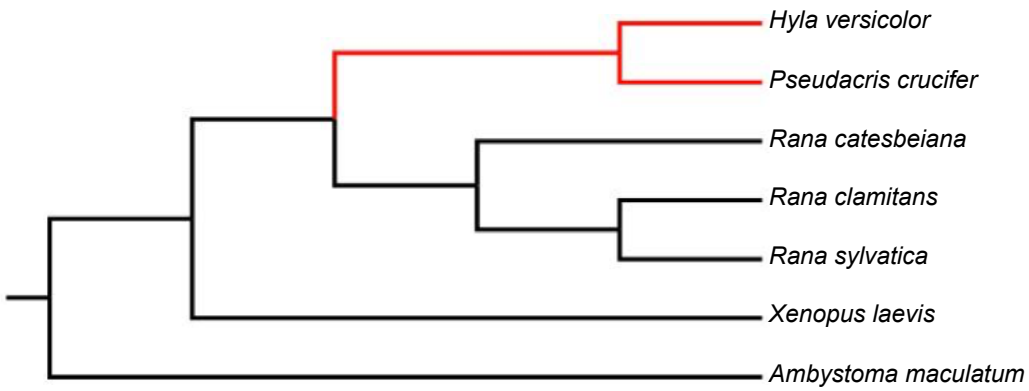
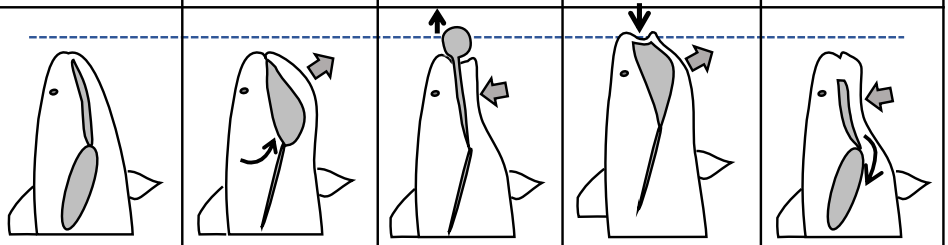
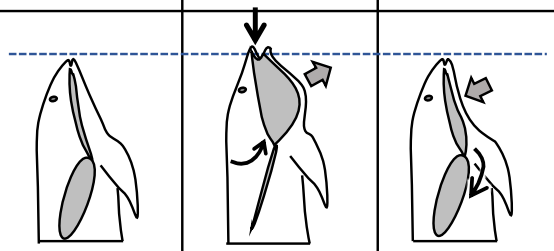
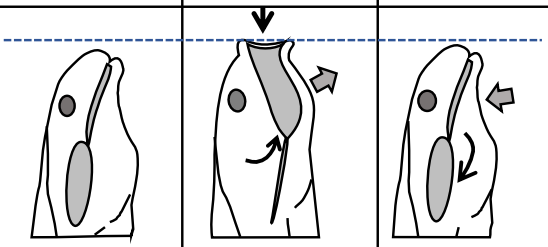
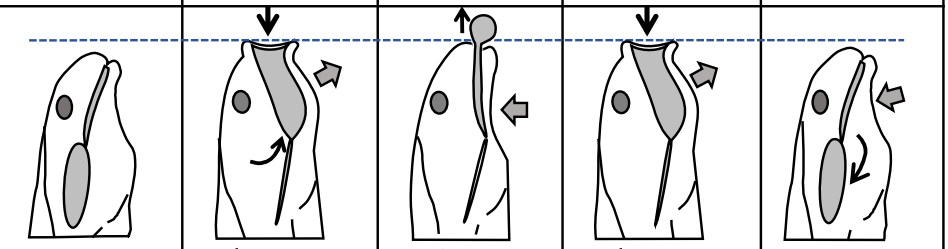
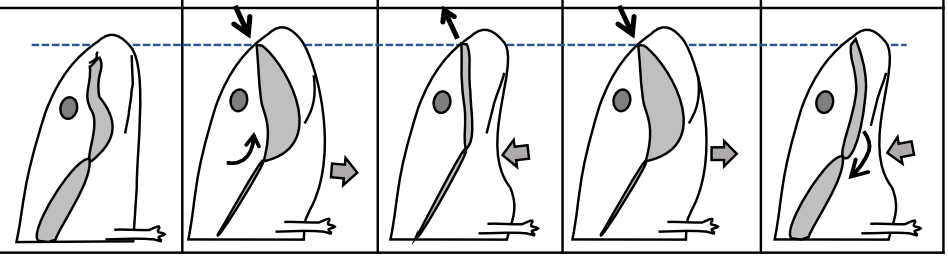
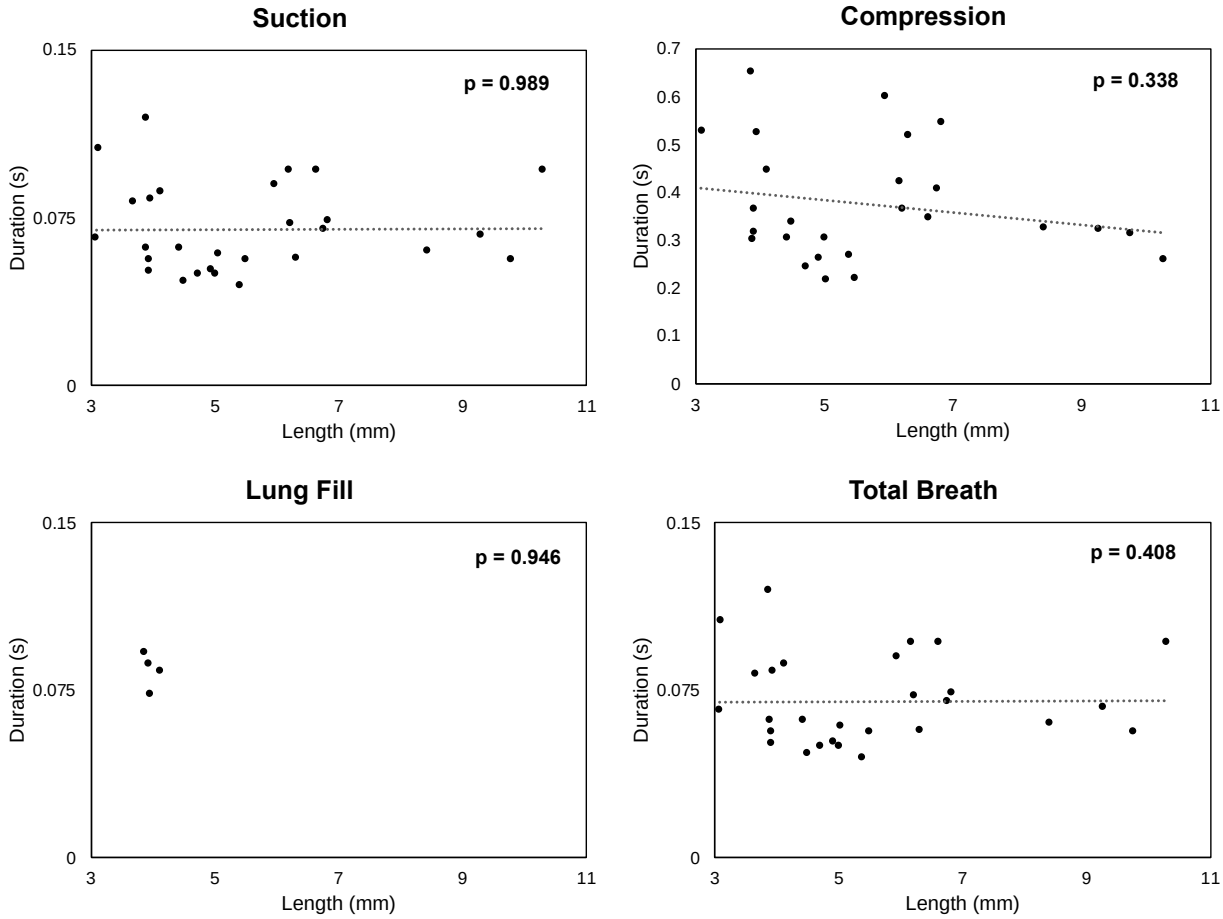

Hyla versicolor
Pseudacris crucifer
Rana catesbeiana
Rana clamitans
Rana sylvatica
Xenopus laevis
Ambystoma maculatum

Figure 1.6: Simplified schematic of two- and four-stroke breathing modes observed across vertebrates. **Row 1**: Four-stroke breathing in an actinopterygian (based on data in Brainerd, 1994. **Row 2**: Two-stroke breathing in a dipnoan lungfish (based on data in Bishop and Foxen, 1968. **Row 3:** Two-stroke breathing (single bubble-sucking) in a hylid tadpole, as described here. **Row 4:** Four-stroke breathing (double bubble-sucking) in a hylid tadpole, as described here. **Row 5:** Four-stroke breathing in the aquatic salamander *Amphiuma* (modified from Simons et al., 2000). Thick, gray arrows indicate bucco-pharyngeal movements (expansion and contraction) and thin, black arrows indicate the direction of airflow into and out of the lungs and buccal chamber.

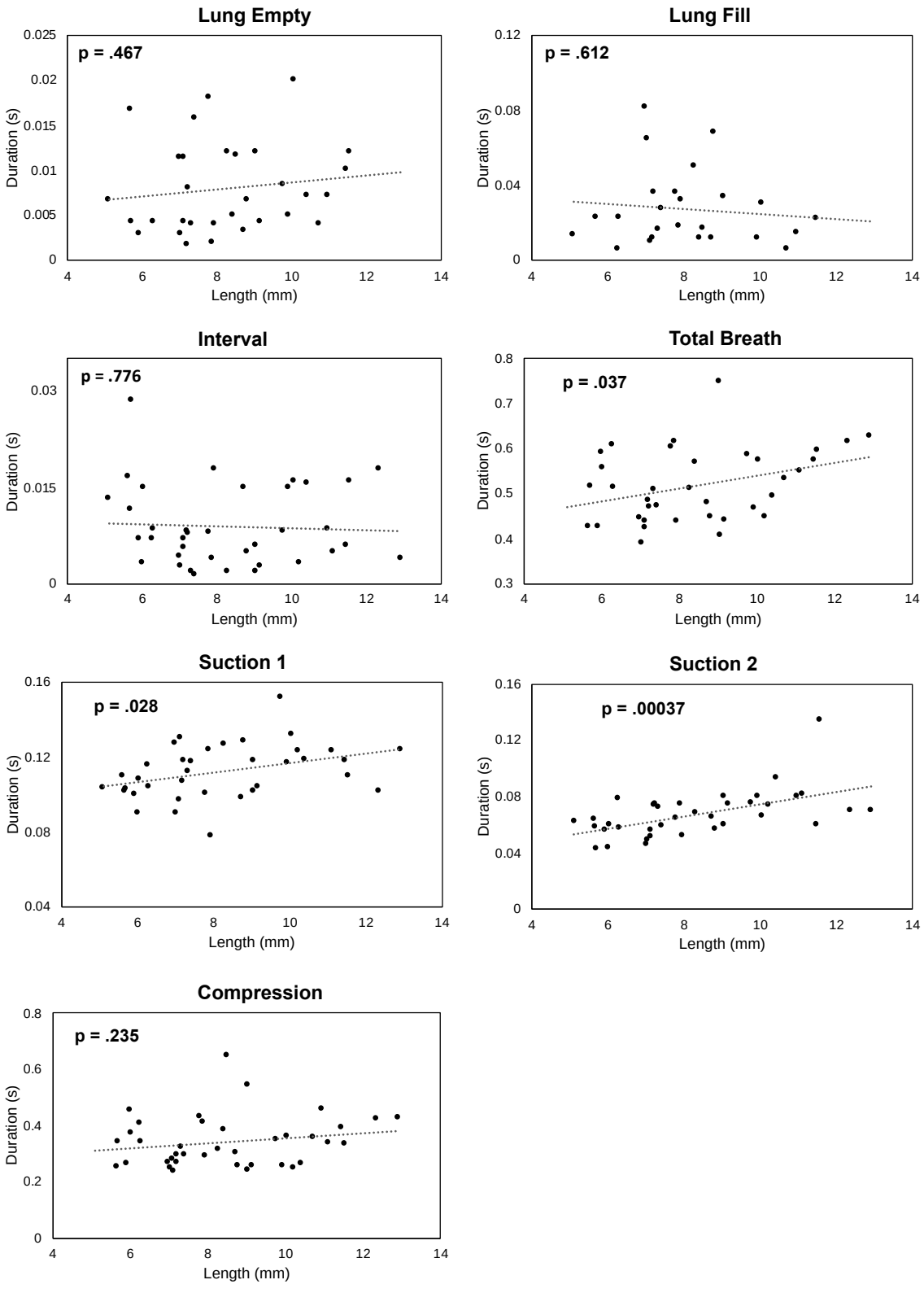
Stroke no. :	0	1	2	3	4
Actinopterygia					
Dipnoi					
Hyla (early larva)					
Hyla (later larva)					
Amphiuma					

Supplemental Figures

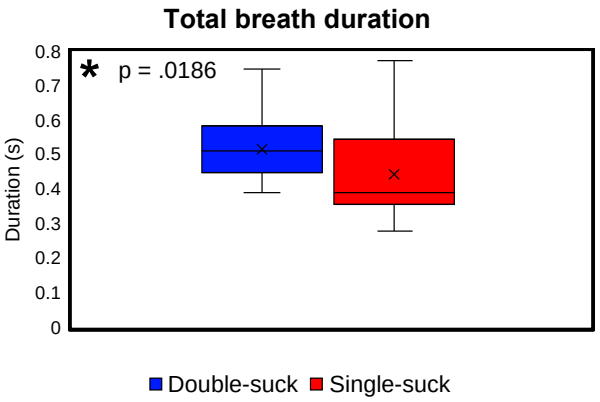
Supplemental Figure 1.1: Ontogenetic trends in the durations of kinematic events (Single Bubble-sucking).

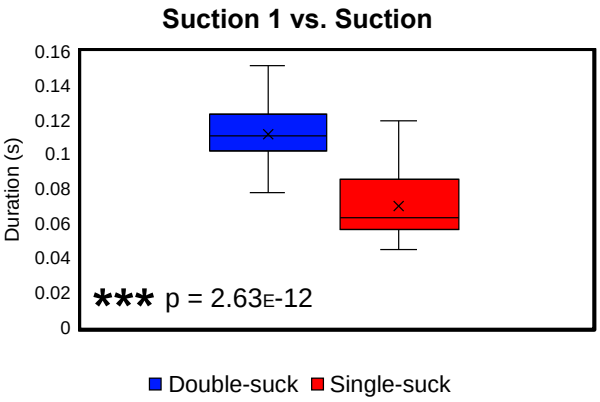

Suction
p = 0.989
Duration (s)
Length (mm)
Compression
p = 0.338
Duration (s)
Length (mm)
Lung Fill
p = 0.946
Duration (s)
Length (mm)
Total Breath
p = 0.408
Duration (s)
Length (mm)

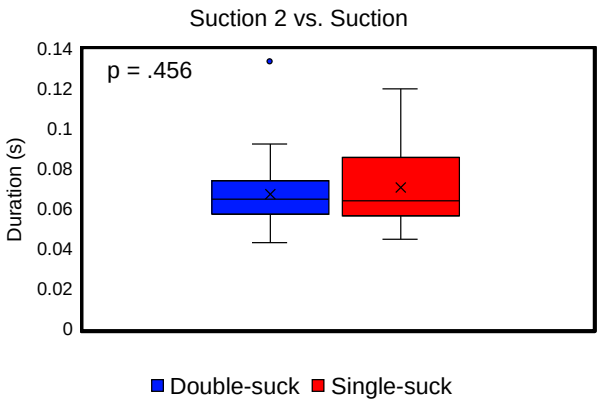
Supplemental Figure 1.2: Ontogenetic trends in the durations of kinematic events (Double Bubble-sucking).

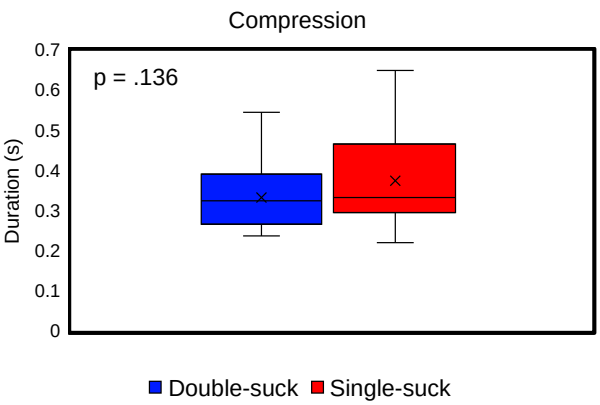

Lung Empty
p = .467
Duration (s)
Length (mm)
Lung Fill
p = .612
Duration (s)
Length (mm)
Interval
p = .776
Duration (s)
Length (mm)
Total Breath
p = .037
Duration (s)
Length (mm)
Suction 1
p = .028
Duration (s)
Length (mm)
Suction 2
p = .00037
Duration (s)
Length (mm)
Compression
p = .235
Duration (s)
Length (mm)

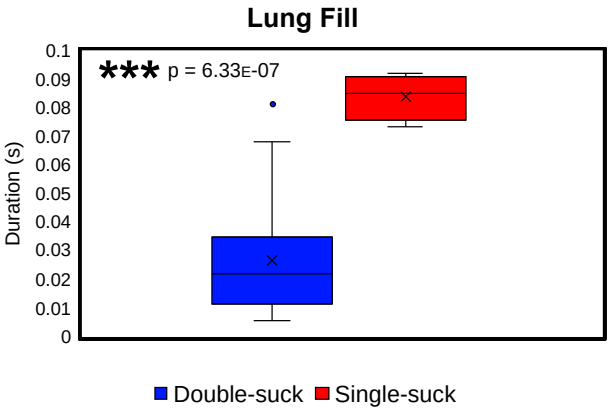
Supplemental Figure 1.3: Comparisons of the kinematic events between single and double bubble-sucking.


Total breath duration
* p = .0186
Duration (s)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Double-suck
Single-suck


Suction 1 vs. Suction
*** p = 2.63E-12
Duration (s)
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Double-suck
Single-suck


Suction 2 vs. Suction
p = .456
Duration (s)
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Double-suck
Single-suck


Compression
p = .136
Duration (s)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Double-suck
Single-suck


Lung Fill
*** p = 6.33E-07
Duration (s)
0.1
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
Double-suck
Single-suck

Chapter 2

Phylogenetics of Lung Loss in Anuran Larvae and the Implications for Toad Evolution (Anura: Bufonidae)

ABSTRACT

The loss of lungs in tetrapod vertebrates is an uncommon phenomenon, having occurred only a handful of times across amphibians and no other tetrapod group. Lung loss in salamanders is often associated with species that live in fast-flowing streams, which is reasonable in the context of both respiration and locomotion. The absence of lungs in the tadpole stage of frog development has also been tied to a stream habitat, although this connection has never been confirmed phylogenetically. I examine the phylogenetic patterns of lung loss and larval habitats across anurans and test for a correlation between the two traits, finding that lung loss in anuran tadpoles is strongly correlated with a stream-dwelling larval habitat. However, the true toads (Anura: Bufonidae) consist of mostly pond-breeding frogs, despite lacking larval lungs. I hypothesized that a common ancestor of modern bufonids lost larval lungs in a stream habitat and then transitioned back onto land, thereby explaining the modern distribution of larval lunglessness across the Bufonidae. The results of my phylogenetic analyses do not support this hypothesis, however, and instead more strongly support the alternative hypothesis, that lungs were lost in a common ancestor of bufonids associated with a lentic, larval habitat.

INTRODUCTION

The diversity of form and function in amphibian respiratory systems suggests many questions from diverse scientific perspectives. In general, there are three methods of gas exchange potentially available to adult and larval amphibians: aquatic breathing with gills, aerial breathing with lungs, and cutaneous gas exchange in either medium. There is significant interspecific variation in respiratory strategies among amphibians and in addition, there is often variation across an individual's ontogeny (e.g., West and Burggren, 1982; Feder, 1984). Anuran amphibians (frogs), in particular, are excellent examples of amphibians with extreme ontogenetic shifts in respiratory strategies. Adult frogs typically use a combination of lung breathing and cutaneous respiration to fulfill oxygen requirements, while larval frogs (tadpoles), use aquatic gills superficially similar to those of fish while also using cutaneous respiration (West and Burggren, 1982). In addition, many tadpoles have functional lungs during a portion of their development before metamorphosis.

The presence of lungs in anuran tadpoles is somewhat mysterious, as tadpoles have two additional avenues for gas exchange (gill respiration and cutaneous respiration), and so the purpose of lungs is not clear (Feder, 1982). Three hypotheses offer adaptive explanations for the presence of larval lungs in tadpoles. Perhaps the simplest possibility is that tadpole lungs are accessory respiratory structures. Redundancy is common in natural systems, so there is no *a priori* reason to think that lungs would not be useful for gas exchange in a tadpole with other means of performing gas exchange. Beyond this, Wassersug and Feder (1984) have shown the physiological importance of lungs for gas exchange in larval *Xenopus* and *Rana*. Phillips et al. (in prep.) have also shown that larval *Hyla versicolor* have vascular lungs, and display behavior that indicates a respiratory role for air-breathing. A second possible function of lungs in larval

anurans is hydrostatic control. Suspension feeders such as *Xenopus* make clear use of lungs to support themselves in the middle of the water column (Wassersug and Feder, 1983), and Gee and Waldick (1995) showed that *Hyla* and others specifically inflate their lungs to remain neutrally buoyant. A third possibility is that lungs serve a developmental purpose in tadpoles. It is possible that early inflation and development of lungs in larval frogs allows for the final development of lungs in adult frogs. This was supported by Pronych and Wassersug (1994) who found that *Xenopus* tadpoles did not develop lungs and did not successfully metamorphose when they were prevented from breathing air throughout the larval period, even in water with high quantities of dissolved oxygen. These three hypotheses are not mutually exclusive, and it is likely that more than one is applicable either concurrently or at different stages of tadpole development.

Some authors have assumed that most frogs have larval lungs, and so presume that any unknown taxon has lungs as a tadpole (Duellman and Trueb, 1986; Wells, 2007). This hypothesis has not been examined in a phylogenetic context, which I undertake in this study. Despite this general understanding however, there are several well-documented cases in which lungs are absent in tadpoles. Larval lunglessness often occurs in specialized stream-dwelling taxa (see below), which are characterized by a repeated set of morphological characters reflecting adaptations for life in streams (Noble, 1929; Orton, 1953; Wells, 2007). Orton (1953) refers to this ecotype as "mountain brook type" tadpoles and a typical feature of this type is a lack or reduction of larval lungs. Some stream-living tadpoles are suctorial, with large suction-generating structures to cling to rocks in stream torrents, while others are fossorial, burying themselves in substrate at the bottoms of streams (Noble, 1929; Orton, 1953; Wells, 2007). There is also at least one well-documented case in which the absence of larval lungs is not obviously associated with stream ecology: the true toads (family: Bufonidae). Because of this group's high

diversity, not all members have been examined for the presence of lungs. However, every bufonid species that has been examined to date has been found to lack lungs up until metamorphosis, and so several authors have suggested that no bufonid tadpole has lungs (Wassersug and Seibert, 1975; Haas, 2003; Wells, 2007).

Most members of Bufonidae are considered "typical toads". These frogs are dry to the touch as adults, have relatively short larval periods and are explosive breeders that lay thousands of eggs at once in long strings (Duellman and Trueb, 1986; Altig and McDiarmid, 1999; Wells, 2007). These taxa are ecologically convergent with other non-bufonid frogs such as *Scaphiopus*, *Pelobates*, and *Microhyla*, among others. However, all of these taxa inflate their lungs as tadpoles, while bufonids do not. Typical bufonids breed in lentic bodies of water that likely face the possibility of anoxia. Noland and Ultsch (1981) found that the typical toad *Anaxyrus terrestris* preferentially sought out more oxygenated microhabitats within ponds, and Feder (1983a) found that toad tadpoles are less tolerant of anoxia than similarly sized tadpoles of lunged (non-bufonid) species. Toad tadpoles are probably less able to deal with anoxia due to their lack of lungs, so it would seem unlikely that selection would have led to lung loss in a lunged, pond-dwelling ancestral species living in a lentic environment.

There are also several other bufonid morphotypes seen across toads that depart from the "typical toad" life history. Some specialize in streams, particularly in the South American and Asian tropics. These taxa, including the well-known genus *Atelopus*, live in fast-flowing streams and have specialized suctorial structures to cling to rocks (Duellman and Lynch, 1969). These taxa are morphologically convergent with other non-bufonid stream-dwelling taxa (see above). For these groups, absence of larval lungs makes functional sense, for the reasons outlined below. There are some toads that exhibit direct development and skip the larval phase entirely. These

toads include genera such as *Nectophrynoides*, *Oreophrynella*, among others. Direct-development has evolved many times across anurans (Gomez-Mestre et al., 2012), and apparently many times within bufonids as well (Van Boxclaer et al., 2010). Finally, several bufonid groups consist of breeding specialists with free-living tadpoles that live in neither ponds nor streams. Some of these taxa are terrestrial (*Dendrophryniscus*), while others breed in phytotelms, such as in bromeliads or other small, sheltered bodies of water. These phytotelmic species (e.g., *Nectophryne*, *Pelophryne*) lay small numbers of eggs in small, shallow bodies of water typically in tropical rainforests (Lehtinen et al., 2004). Water bodies such as bromeliads are often subject to high temperatures, are sheltered from wind (preventing mixing with the air) and shaded from the sun (preventing photosynthetic activity) (Laessle, 1961). All these factors lead to a dangerous situation for a tadpole due to anoxia, and outside of bufonids, frogs that breed in such water bodies have adaptations to deal with this problem. Eggs of the microhylid genus *Hoplophryne* are laid in bamboo axils in the African tropics and the tadpoles have evolved to deal with anoxia by losing their gills and exaggerating their lungs (Noble, 1929). If the diffusion gradient is too extreme, gills can become costly and oxygen will actually be lost in these microhabitats. If possible, it would seem advantageous for toads living in such environments to re-evolve lungs in their larval phase.

All examined bufonid species lack lungs as larvae, despite the fact that lungs should be advantageous in pond-dwelling species (e.g., *Anaxyrus*, *Rhinella*, *Bufo*) and especially, phytotelmic species (e.g., *Nectophryne*, *Pelophryne*). Why then, is the absence of lungs seemingly fixed in toads? In other frogs, the presence or absence of lungs appears to be mostly defined by ecology. Many, although not all, stream-breeding taxa have been shown to lack lungs as tadpoles, while other species maintain lungs if living under conditions in which they are useful

(such as in a typical pond-dwelling species), or necessary (in a phytotelm-dwelling species) (Wassersug and Heyer, 1989). In toads however, this is not the case – all species apparently lack lungs, regardless of ecology, as far as is known. Because bufonids encompass so much reproductive diversity, there is no single adaptive explanation that explains the maintenance of this trait across evolutionary time. Instead, the apparent uniform lack of lungs across bufonids is best explained by historical contingency. This theory supposes that some proto-bufonid ancestor had a phenotype that made the loss of larval lungs advantageous, and from that point forward this condition has been maintained by some form of developmental constraint. It is important to acknowledge that all adult toads retain functional lungs. Strictly speaking, lungs are not "lost" in bufonids, and so invocations of Dollo's Law (i.e., that re-evolution of complex phenotypes is extremely rare - Gould, 1970) are misleading. It is possible, however, that there are functional constraints on larval development such that once the timing of lung development is "pushed back" towards metamorphosis, it cannot be brought forward again without disturbing other developmental processes, making a reversal impossible in the short term.

When trying to understand why some frogs lack lungs as tadpoles, many authors have pointed out the previously noted correlation between streams and lunglessness (Noble, 1929; Wassersug and Heyer, 1989; Altig and McDiarmid, 1999; Wells, 2007; Gee and Waldick, 2012). Streams are constantly oxygenated by the mixing of water, and so perhaps stream-dwelling taxa have no need of lungs for gas exchange, as they are probably able to survive on gill and cutaneous respiration (Noble, 1929). In addition, it is possible that the hydrostatic advantages that lungs provide tadpoles in lentic water-bodies may be deleterious in streams. Inflated lungs make tadpoles more buoyant, which makes them more at risk of being swept away in fast-flowing streams (Wilder and Dunn, 1920; Wake, 1966). Bruce et al. (1994) showed that when

larval *Ambystoma* were prevented from inflating lungs, they outperformed their lunged counterparts in swimming trials held in lotic conditions. Gee and Waldick (2012) compared lungless, stream-dwelling tadpoles to lunged, pond-dwelling species, and also came to the conclusion that lunglessness was derived in stream-dwelling groups to improve locomotor performance. These results have cumulatively led to a general understanding that the selection imposed by a stream environment leads to reduction and loss of larval lungs in tadpoles (Wassersug and Heyer, 1989; Wells, 2007).

This same line of reasoning has also been used to explain lunglessness in plethodontid salamanders. There are several, repeated cases of lung loss or reduction across salamanders, and most occur in stream-dwelling species, with the exception of a large, homogeneously lungless group: the Plethodontidae. Within plethodontids, there are many different ecologies, some associated with streams, but most not, instead having totally terrestrial life-cycles. Wilder and Dunn (1920) first suggested that the origin of plethodontid lunglessness can be traced back to a stream-dwelling ancestor of all plethodontids, and that once lungs were lost, plethodontids were incapable of re-evolving that complex trait. Thus, lunglessness in Plethodontidae was historically the result of adaptive selection due to their stream habitat, but the current distribution of the trait is due to historical contingency once a member of the group moved out of streams. Others have disputed this hypothesis, arguing that geological evidence suggests that streams were not available while this group was evolving (Ruben and Boucot, 1989). More recently, this hypothesis has regained traction, especially given the fact that several of the early branching members of the group live in streams (Beachy and Bruce, 1992). If larval lunglessness is a fixed trait in bufonids, then perhaps these same arguments can also be applied to explain larval lunglessness in toads. If some ancestor of toads bred in streams, then we would expect a fairly

high chance of lung loss in tadpoles under that selective regime. After lunglessness becomes fixed in that lineage, then it would only require a single transition back into a pond ecology to explain the large number of pond-dwelling bufonids.

In this study, I assess the distribution of lungs across as many larval anurans as possible, in order to test the hypothesis that the evolution of larval lunglessness is correlated with a lotic life history. I also use parsimony, likelihood, and a dependent model of trait evolution to test the hypothesis that the most recent common ancestor of the Bufonidae bred in a stream and so lost larval lungs in response to the adaptive pressures imposed by a lotic environment.

MATERIALS AND METHODS

Data collection

Lung presence/absence data was collected for as many species as possible, primarily from the literature. In some species the presence/absence of lungs was confirmed via dissection, and in others the presence of lungs was inferred by the reported occurrence of air-breathing behavior. In total, I was able to find reliable evidence of lung absence/presence for 160 species, of which 156 could be reliably included in a phylogenetic analysis. Life history information was collected for all 160 species from the databases Amphibiaweb and the IUCN redlist (Amphibiaweb, 2019; IUCN, 2019). Larval habitat was recorded as either lentic or lotic. Using these two categories simplifies the biological realities of reproductive diversity, but is necessary for the discrete models I used to model trait evolution (see below). I scored any description of breeding location that referred to a pond, pool, puddle or similar, as lentic, and any description that referred to a stream, torrent, rapid or rocky surfaces associated with such environments, as lotic. The data for these taxa are presented in Table 2.1.

Phylogenetic Trees

I used a time-calibrated ultrametric tree of over 2,000 amphibian species for all analyses (Pyron and Wiens, 2013). This tree was selected because it has some of the best taxon sampling of any available tree and so could accommodate my dataset. In several cases, specific species in the dataset were not included in the Pyron and Wiens (2013) tree while their congeners were. In these cases, I assumed that genera are monophyletic and substituted the tip of an included member of that genus for the species for which lung data is available. In (four) cases, there was no reliable way to include taxa on trees, so these taxa were excluded from the analysis, reducing the sample from 160 to 156. I trimmed the starting tree (Pyron and Wiens, 2013) down to the 156 taxa using the package ape v.5.0 in R v.3.4.3 (Paradis and Schliep, 2018; R Core Team, 2017). This first tree includes 156 taxa and is henceforth referred to as Tree 1. The sample of taxa for which lung data is available is far from complete, and my dataset only includes nine (of ~ 35) genera of toads. To combat this problem, I created a second tree to include as much bufonid diversity as possible. This second tree is henceforth referred to as Tree 2. Tree 2 includes the original 156 taxa for which lung data is available, in addition to nearly all bufonids included in the original 2,000+ taxa tree (Pyron and Wiens, 2013), bringing the total number of taxa to 335. All bufonids were assumed to lack lungs as larvae, and breeding biology for these added bufonids was collected in the same manner as explained above. The earliest branching genus of bufonids, *Melanophryniscus*, presents a different problem than merely lack of sampling. This group includes both stream- and pond-breeding members, and yet the only member of the group that has been examined for lung presence happens to breed in streams (*M. orejasmirandai*), while only pond-dwelling members have been included in previous molecular phylogenetic analyses (e.g., Pramuk, VanBoxclaer, Pyron and Wiens). To deal with this potential problem, I

chose to use only two members of *Melanophryniscus* in Tree 2: one pond-breeding member, (*M. stelzneri*), and one stream-breeding species (*M. orejasmirandai*).

Trait Correlation Analysis

I used the program BayesTraits Version 3.0.1 (Pagel, 1994; Meade and Pagal, 2019) to assess the hypothesized trait correlation between larval habitat and larval lunglessness. This was done with paired analyses using dependent and independent models of phenotypic evolution to test if lungs are indeed correlated with breeding biology. Independent analyses use four rates to model changes between stream and pond as well as between lunged and lungless, while dependent analyses use 8 rates, considering the effects that traits may have on one other. I restricted the rates of regaining lungs to zero in both the dependent and independent analyses to prevent models from pursuing unrealistic explanations of the data, but otherwise left all rates with flat priors from 0 to 100. The MCMC function was used to estimate rate matrices and the ancestral states of the most recent common ancestor of bufonids for every run. Trees were scaled by .001 as recommended in the BayesTraits manual. A stepping stones analysis was used (Xie et al., 2011), with 100 stones every 1000 iterations to calculate marginal likelihoods of each run, and a Bayes Factor test was used to compare the dependent and independent models (Gilks et al., 1996). Several taxa were fossilized to help the model find a biologically reasonable explanation for data. The most recent common ancestor (MRCA) of all frogs was fossilized as pond-breeding and lunged and the MRCA of typical toads (formerly of the genus *Bufo*) was fossilized as pond-breeding and lungless (see Fig. 2.1). These analyses were performed on both Tree 1 and Tree 2 to assess whether adding more bufonid taxa changed the results.

Ancestral State Reconstructions

I first used parsimony and likelihood in Mesquite to estimate the number of independent evolutions of lunglessness across anurans. I then used four methods to estimate the ancestral state of larval habitat for the MRCA of all bufonids: a) parsimony in Mesquite, b) maximum likelihood in mesquite, c) independent model analysis in BayesTraits, d) dependent model in BayesTraits. Mesquite analyses were done under Mesquite V 3.51 using built-in character history traces (Maddison and Maddison, 2019). BayesTraits analyses were done as described above, except that ancestral states were calculated using the addnode command. Another model was also tested that assumed that lungs were always lost in larval habitats associated with streams by running the above-described dependent model with the rate of losing lungs in ponds set to 0. Again, all ancestral-state reconstructions were done on trees with limited (tree 1) and more complete (tree 2) inclusion of toads in order to assess the effect of adding more bufonid taxa to the analysis.

RESULTS

Distribution of Larval Lunglessness across Frogs

Table 2.1 presents larval habitat as well as lung presence/absence data for 160 taxa. An asterisk (*) is used to denote tadpoles for which the presence of lungs has not been confirmed by dissection, but instead has been inferred by the observation of air-breathing behavior.

I found that the presence of larval lungs is extremely common across frogs. Most groups are made up entirely of species with lunged taxa. Larval lunglessness has evolved many times independently across anurans. Under parsimony, it has evolved 16 times, and under likelihood, it has evolved 17 times. The conflict between these results is due to the MRCA of *Ascaphus* and

Leiopelma, which likelihood suggests had lungs and bred in ponds, and so lunglessness evolved independently in each genus according to likelihood but not parsimony (Fig. 2.1). Most instances of lungless tadpoles are stream-dwelling taxa, but there are two groups that contain lungless members with a pond-associated larval habitat and life history. As discussed above, one of these instances is the Bufonidae, of which none examined were found to possess to larval lungs. The second is a group of myobatrachid frogs that includes both stream-dwelling (*Mixophyes* and *Taudactylous*)and pond-dwelling members (*Crinia*, *Pseudophryne*).

Trait Correlation

My results indicate that there is a strong correlation between the presence or absence of larval lungs and larval habitat across frogs. Using Tree 1, I found that the marginal likelihood (as estimated using the stepping stones method) of the dependent model is -146.02, while the marginal likelihood of the independent model is -210.75. Using the method described by Gilks et al. (1996) I determined there is very strong evidence of correlation by calculating a Bayes Factor of over 100 in support of the more complex, dependent model (Fig. 2.2). When more bufonids were added to the analysis in Tree 2, the correlation does not disappear, and in fact the Bayes factor test gives an even stronger result (Fig. 2.2). The rate matrices produced by the dependent models for both trees suggest that the rate of lung loss is roughly one order of magnitude higher in streams than ponds (Fig. 2.2). However, model testing does not support a simplified dependent model that sets the rate of losing lungs in a pond to zero (table 2.2). The rate matrices produced for trees 1 and 2 are similar, with some differences in rates of change from pond to stream and stream to pond while lungless (Fig. 2.2).

Ancestral State Reconstruction

No method used unilaterally predicted a stream-breeding MRCA of modern bufonids. The analysis most favorable to a stream-breeding MRCA is parsimony, under which both a stream-breeding and a pond-breeding ancestor are equally likely (in both trees). Likelihood on the other hand, gave a 97% probability of a pond-breeding MRCA of bufonids in tree 1 and a 99% probability of a pond-breeding MRCA in tree 2. The independent analyses also supported a pond-breeding MRCA for bufonids with a 63% probability of a pond-breeding ancestor in tree 1 and 85% chance in tree 2. The dependent analysis, despite suggesting a higher rate of losing lungs in the stream than a pond, gave 73% chance of a pond-breeding MRCA in tree 1 and 95% chance in tree 2. When the rate of losing lungs in ponds was set to 0, the model nevertheless predicted a pond-breeding MRCA of bufonids in both trees (67% in tree 1; 90% in tree 2). All values are presented in table 2.2.

DISCUSSION

The phylogenetic distribution of larval lunglessness across anurans is noteworthy for several reasons. Despite the nearly twenty inferred independent losses of larval lungs, a vast majority of taxa with lungless larvae are likely to be in one family: the Bufonidae. Because nearly every group lacking lungs is composed exclusively of stream-dwelling members, one might expect that most lungless tadpoles live in streams, especially given the historical attention that has been paid to lung loss in stream environments. Instead, due to the large number of bufonids, purely by numbers of species, there are almost certainly far more pond-dwelling frog species than stream-dwelling species that lack lungs. Tree 1 does not show this reality properly, as only a few bufonid species have actually been examined for the presence of larval lungs. Tree 2 makes an

attempt to reflect this reality, although having bufonids make up nearly half the total taxa is also somewhat misleading.

My results suggest that there are at least 16 independent evolutions of larval lunglessness across frogs. I found that under likelihood, monophyletic lineages entirely composed of members lacking larval lungs represented single, independent evolutions of larval lunglessness, except in the case of *Leiopelma* and *Ascapus*. Instead, likelihood predicts that the MRCA of these genera had larval lungs and bred in ponds. These two ancient frog genera are likely relict taxa and the only extant members of groups that were once much larger and more diverse. They are each on long branches and in very different biogeographic regions of the world. For all these reasons, I interpret these two stream-dwelling, lungless lineages as having independently evolved both a stream-oriented biology as well as larval lunglessness, especially given that that their tadpole morphology is divergent from one another. *Leiopelma* is a genus consisting of mostly direct developing species (no larval stage) except for the one taxon included in the analysis. *Ascaphus* is highly derived for living in streams with a body-sucker and other stream adaptations that *Leiopelma hochstetteri* lack (Bell and Wassersug, 2003). Beyond these two lineages, there are stream-adapted taxa that lack lungs within many different major groups of frogs. Outside the Bufonidae, I found one other group of lungless tadpoles that are not obviously associated with streams. This group is nested within the Myobatrachidae and includes several stream-dwelling members as well as two members that live in ponds or similar lentic water bodies: *Pseudophryne bibroni* and *Crinia tasmaniensis* (Fig. 2.1). *Pseudophryne* are particularly interesting because, as the name suggests, they are superficially similar to bufonid toads. This group is much larger than my sample suggests and contains many additional pond-breeding species for which no data on larval lungs exists. Currently, the placement of stream-dwelling members at the base of these

lungless myobatrachids suggests a stream-dwelling ancestor for the group, but without better taxon sampling any further interpretation would be speculative at best.

As previous authors have noted, (e.g. Noble, 1929; Wassersug and Heyer, 1989), not all tadpoles that live in streams lack lungs. This is further supported by a phylogenetic perspective. There are many stream-breeding frogs with stream-adapted tadpoles that retain larval lungs, which can be interpreted in several different ways. Perhaps these taxa deal with the disadvantages of having lungs in a lotic environment by avoiding currents behaviorally. Alternatively, water temperature is likely to play a large role in whether or not lungs are physiologically important for respiration. Cold, mountain streams seem particularly likely to produce lungless tadpoles (e.g. *Ascaphus*) owing to mixing and the capacity of cold water to hold more oxygen, while warmer, tropical streams often produce lunged tadpoles (e.g. *Hypsiboas heilprini* (Noble, 1929)). Lacking data on relative stream temperatures, I am unable to test that hypothesis here, but future studies should address this question in a phylogenetic framework.

As expected, I found strong evidence for a trait correlation between breeding in streams and lacking lungs in the larval stage. Bayes factor model selection chose the dependent model over the independent model in tree with both limited and more extensive representatives of bufonids (trees 1 and 2). The dependent models give a much higher rate of losing lungs in streams than in lentic water bodies, as expected. Given these results, I was surprised to find that no analysis strongly supported the possibility of stream-dwelling ancestor for bufonids. Parsimony suggests that a stream-dwelling ancestor is plausible, but no other analysis gives more than a 40% chance of a stream-dwelling MRCA for bufonids. Among the methods that I used to estimate the probability that the MRCA of bufonids had stream-adapted tadpoles, only the dependent model considers the fact that bufonid tadpoles are lungless. The other models of

evolution ignore the potential relationship between a stream environment and larval lunglessness. For this reason, I had expected the dependent model of trait evolution to be more inclined toward to a stream ancestor for bufonids, and so I was surprised that the dependent model actually gave a lower probability of a stream ancestor than the independent model, which treats lunglessness and breeding biology independently.

All analyses performed here are consistent with the possibility that the MRCA of toads did not breed in streams as hypothesized and instead bred in ponds as many modern toads do. The results provide no direct evidence supporting a stream-breeding MRCA, and instead the results of several independent analyses all converge on a pond-breeding MRCA for toads. Assuming that this result is true, there are two potential biological explanations for how and why modern toads lack lungs as tadpoles. It is possible that the loss of lungs in bufonid tadpoles might still have evolved in a stream-dwelling ancestor, regardless of the state of the most *recent* common ancestor of toads. This would be possible if a shift to stream-breeding, followed by a subsequent loss of larval lungs occurred early on the stem leading to modern bufonids, and was followed by a reversal that returned the lineage back to pond-breeding before the MRCA of bufonids and the divergence of the branch that leads to *Melanophryniscus.* While speculative, our analyses do allow for this possibility. When the rate of losing lungs in ponds is manually restricted to 0, the dependent model described above still predicts that the MRCA of bufonids breeds in ponds, not streams. While unparsimonious, it is possible that even if the MRCA of toads bred in ponds, larval lunglessness in toads evolved in an earlier, stream-breeding ancestor.

Alternatively, perhaps no toad ancestor had stream-adapted tadpoles and toads lost larval lungs for reasons entirely independent of a stream ecology. Toads have some of the shortest larval periods across frogs, so perhaps the loss of lungs helped to shorten the larval life phase,

allowing toads to escape the water faster, as suggested by Wells (2007). Unfortunately, there is no way to polarize the causality of this reasonable connection. Perhaps the lack of lungs in bufonid tadpoles created evolutionary pressures that forced bufonid tadpoles to get out of the water faster as they are less able to deal with anoxia than lunged taxa (Feder, 1983a). One might examine the duration of the larval period across toads to see if this trait is consistent across taxa such as *Rentapia* or *Atelopus*, stream-dwelling bufonid taxa that would presumably face no such pressures, but these data are not readily available. A clue to this question can be found in other taxa with shortened larval periods, such as the non-bufonid spadefoot toad *Scaphiopus*, which has the shortest known free-living tadpole stage of any frog (Newman, 1987, 1988). All known scaphiopodids however, including *Scaphiopus*, have well-developed lungs as larvae, showing that the larval period can be extremely abbreviated without the loss of larval lungs. This fact does not disprove the possibility that lung-loss might have been selected for in toads breeding in still water to shorten the larval period. However, the paucity of lungless taxa in non-bufonid groups that breed in temporary pools works against this, with the exception of the myobatrachids *Pseudophryne* and *Crinia*, and these genera are not known for having short larval periods (Amphibiaweb, 2019).

That *Pseudophryne* and *Crinia* lack larval lungs is a very unexpected because these frogs are not stream adapted and do not have short larval periods. As in bufonids, perhaps the best way to explain their lack of larval lungs is historical contingency. The phylogenetic arrangement of the members of this group available for this study strongly suggests a secondary evolution of pond-breeding from a stream-breeding, lungless ancestor (Fig. 2.1). However, better taxon sampling is needed to confirm this pattern. If true, this group would provide some evidence for an evolutionary transition from a lungless, stream-breeding frog to a lungless, pond-breeding

frog analogous to my hypothesized evolutionary sequence for bufonid toads. What is particularly noteworthy about the lack of larval lungs in *Pseudophryne* is the degree of phenotypic convergence between the adult phenotypes of these frogs and typical bufonid toads. They are both dry-skinned, pond-breeding frogs with alkaloid toxins in their skin (Daly et al., 1990). It would seem likely that these shared independent larval and adult convergences are not pure coincidence, and yet it is unclear whether the lack of larval lungs might be a cause or a symptom of adult convergence in the two groups.

It is possible that toads did not evolve in streams at any point along the branch leading to crown bufonids. It is even possible that an ancestral species was stream-dwelling at some point, but that lung loss did not occur in streams. I do not present any quantitative evidence to dispute the possibility that long loss in bufonid larvae is unrelated to a lotic environment, except that there is no satisfying adaptive explanation for why larval lung loss would have occurred outside a lotic environment. Despite this, the results most strongly support the conclusion that the most recent common ancestor of bufonids did not breed in a stream, and instead the tadpoles lived in still water. Under this scenario, the hypothesis that larval lung loss in bufonids was related to a stream-dwelling ancestor is unparsimonious, but not impossible. Lung loss could have occurred earlier along the stem in streams and then transitioned back into a pond before the divergence of the branch containing *Melanophryniscus.* However, my results most strongly suggest that despite the functional and phylogenetic correlation between a lotic environment and lung loss, larval lung loss in bufonids likely occurred in a lentic habitat.

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TABLES

Table 2.1. Life history and Larval lung presence in anuran tadpoles. An (*) denotes taxa for which larval lungs are inferred by the presence of air-breathing behavior. All breeding biology information was obtained from Amphibiaweb or the IUCN redlist databases and sources for lung presence are indicated by numbers following species names (Amphibiaweb, 2019; IUCN, 2019). Sources in numerical order as they appear in the table below: (Haas, 2003[1]; Schwenk and Phillips, in press.[2]; Rong-chuan et al., 2010[3]; Noble, 1929[4]; Bell and Wassersug, 2003[5]; Gee and Rondeau, 2012[6]; Tu et al., 1999[7]; Feder, 1983b[8]; Kruger and Richter, 1995[9]; Branch, 1983[10]; Caldwell, 1989[11]; Lajmanovich et al., 2003[12]; Marian et al., 1980[13]; Lannoo et al., 1987[14]; Noland and Ultsch, 1981[15]; Dias, in lit.[16], Nodzenski et al., 1989[17]; Wassersug and Heyer, 1989[18]; Wassersug and Seibert, 1975[19]; Haas and Richards, 1998[20]; Feder, 1984[21]).

Species	**Larval Habitat** 1 = lentic 0 = lotic	**Presence of Larval lungs** 1 = lunged 0 = lungless
Adenomera marmorata[19]	1	1
Agalychnis callidryas[1]	0	1
Alsodes monticola[19]	1	1
Alsodes sp.[19]	1	1
Alytes obstetricans[1]	0	1
Amolops ricketti[4]	1	0
Anaxyrus americanus[2]	0	0
Anaxyrus terrestris[15]	0	0
Anaxyrus woodhousii[20]	0	0
Aplastodiscus perviridis[1]	0	1
Ascaphus truei[1]	1	0
Atelognathus reverberii[19]	1	1

Atelopus tricolor[1]	1	0
Barbarophryne brongersmai[1]	0	0
Batrachyla taeniata[19]	1	1
Bombina maxima[1]	0	1
Bombina orientalis[1]	0	1
Bombina variegata[1]	0	1
Bufo bufo[1]	0	0
Calyptocephallela gayi[19]	1	0
Ceratophrys aurita[19]	0	1
Ceratophrys ornata[1]	1	1
Chiromantis xerampelina[1]	0	1
Cochranella granulosa[1]	1	1
Crinia tasmaniensis[19]	0	0
Crossodactylodes sp.[19]	0	1
Crossodactylus gaudichaudii[19]	0	1
Crossodactylus schmidti[1,19]	1	1
Crossodactylus sp.[19]	?	1
Cycloramphus stejnegeri[19]	0	0
Dendrobates tinctorius[1]	0	1
Dendropsophus ebraccatus[1]	0	1
Discoglossus galganoi[1]	0	1
Discoglossus pictus[1]	0	1
Duttaphrynus melanostictus[1]	0	0
Dyscophus antongilii[1]	0	1
Elachistocleis bicolor[1]	0	1
Engystomops petersi[11,19]	0	1
Epipedobates tricolor[1]	0	1
Euphlyctis cyanophlyctis[13]	0	1
Gastrophryne carolinensis[1]	0	1

Gastrotheca riobambae[1]	0	1
Hadromophryne natalensis[1,19]	1	1
Hamptophryne boliviana[1]	0	1
Heleioporus sp.[19]	?	1
Heleophryne rosei[4]	1	0
Hemisus sudanensis[1]	0	1
Hoplophryne rogersi[4]	0	1
Hoplophryne uluguruensis[4]	0	1
Hyla annectans[1]	0	1
Hyla cinerea[1]	0	1
Hyla versicolor[2]	0	1
Hylodes c.f. aspersus[19]	1	0
Hylodes meridionalis[1]	1	1
Hylorina sylvatica[19]	0	1
Hyloscirtus armatus[21]	1	1
Hymenochirus boettgeri[20]	0	1
Hyperolius puncticulatus[1]	0	1
Hypsiboas cordobae[1]	0	1
Hypsiboas geographicus[11 *]	0	1
Hypsiboas heilprini[4]	1	1
Ikakogi tayrona[17]	1	0
Kaloula pulchra[1]	0	1
Kassina senegalensis[1]	0	1
Leiopelma hochstetteri[5]	1	0
Lepidobatrachus laevis[1,19]	0	1
Leptobrachella gracilis[18]	1	0
Leptobrachium hasseltii[1]	1	1
Leptodactylodon boulengeri[17]	1	0
Leptodactylus fuscus[19]	0	1

Leptodactylus gracilis[19]	0	1
Leptodactylus knudseni[19]	0	1
Leptodactylus latinasus[1]	0	1
Leptodactylus mystacinus[19]	0	1
Leptodactylus wagneri[19]	0	1
Leptopelis vermiculatus[1]	1	1
Limnodynastes dumerilii[6]	1	1
Limnodynastes lignarius[19]	0	1
Limnodynastes peronii[1,6]	0	1
Limnodynastes tasmaniensis[19]	0	1
Limnonectes kuhlii[7]	0	1
Limnonectes leporinus[1]	0	1
Litoria genimaculata[1,6]	0	1
Litoria inermis[1]	0	1
Litoria lesueurii[1,6]	0	1
Litoria nannotis[1,6]	1	0
Litoria rheocola[1]	1	0
Macrogenioglottus alipioi[19]	1	1
Mannophryne herminae[1]	1	1
Megaelosia goeldii[19]	1	1
Megophrys montana[1]	1	1
Melanophryniscus orejasmirandai[1]	1	0
Microhyla heymonsi[7]	0	1
Microhyla ornata[7]	0	1
Mixophyes balbus[19]	1	0
Nyctimystes dayi[1,6]	1	0
Odontophrynus achalensis[1]	1	1
Odontophrynus americanus[19]	1	1
Odontophrynus occidentalis[19]	1	1

Odorrana tormota[3]	1	0
Osteocephalus planiceps[1]	0	1
Osteopilus brunneus[14]	0	1
Osteopilus dominicensis[4]	0	1
Osteopilus vastus[4]	1	1
Paa exilispinosa[1]	1	1
Paradoxophyla palmata[1]	0	1
Paratelmatobius lutzii[19]	1	1
Pelobates fuscus[1]	0	1
Pelodytes caucasicus[1]	0	1
Peltophryne peltocephalus[1]	0	0
Phrynomantis bifasciatus[1]	0	1
Phyllobates bicolor[1]	0	1
Phyllomedusa distincta[1]	0	1
Phyllomedusa vaillantii[10 *]	0	1
Physalaemus biligonigerus[1]	0	1
Physalaemus pustulosus[19]	0	1
Pipa carvalhoi[1]	0	1
Platyplectrum ornatus[19]	0	1
Pleurodema borellii [19]	0	1
Pleurodema brachyops[19]	0	1
Pleurodema bufoninum[19]	0	1
Pleurodema cinerea[19]	0	1
Pleurodema kriegi[1]	0	1
Pleurodema nebulosa [19]	0	1
Proceratophrys appendiculata[19]	1	0
Proceratophrys boiei[19]	0	1
Pseudacris crucifer[2]	0	1
Pseudis minuta[1]	0	1

Pseudis paradoxa[1]	0	1
Pseudopaludicola sp.[19]	1	0
Pseudophryne bibronii[19]	0	0
Ptychadena mascareniensis[1]	0	1
Pyxicephalus adspersus[1]	0	1
Rana berlandieri[8]	1	1
Rana catesbeiana[2]	0	1
Rana clamitans[2]	0	1
Rana nigrovittata[1]	0	1
Rana septentrionalis[6]	0	1
Rana sphenocephala[15]	0	1
Rana sylvatica[2]	0	1
Rana temporaria[1]	0	1
Rentapia hosii[1]	1	0
Rhacophorus pardalis[1]	0	1
Rhinella arenarum[1]	0	0
Rhinella marinus[1]	0	0
Rhinoderma darwinii[19]	1	1
Rhinophrynus dorsalis[1]	0	1
Scaphiophryne madagascariensis[1]	0	1
Scaphiopus holbrookii[22]	0	1
Scinax nasicus[12]	0	1
Scinax ruber[1]	0	1
Smilisca baudinii[1]	0	1
Sooglossus sp.[4]	1	1
Spea bombifrons[1]	0	1
Staurois latopalmatus[4]	1	0
Syncope antenori[9]	0	1
Taudactylus diurnus[19]	1	0

Telmatobius jelskii[19]	0	1
Telmatobius marmoratus[19]	0	1
Thoropa petropolitana[19]	1	0
Tomopterna cryptotis[1]	0	1
Trachycephalus resinifictrix[1]	0	1
Xenopus laevis[1]	0	1

Table 2.2 Ancestral state reconstructions and marginal likelihoods of all models. Dependent 2 refers to a dependent model in which the rate of lung loss in ponds is set to 0.

Tree	Model of evolution	Probability[pond ancestor]	Marginal Likelihood (when possible)
Tree 1	Parsimony	0.5	-
	Likelihood	0.97	-
	Dependent	0.73	-146.02
	Independent	0.63	-210.75
	Dependent 2	0.67	-140.68
---	---	---	---
Tree 2	Parsimony	0.5	-
	Likelihood	0.99	-
	Dependent	0.95	-205.87
	Independent	0.86	-300.26
	Dependent 2	0.90	-201.52

FIGURES

Figure 2.1: Phylogenetic Distribution of lunglessness across Anura. Left: Tree 2 with all taxa included. Right: A subset of taxa to allow for easy visualization of the entire anuran tree. Black tips represent species with lunged, pond-living tadpoles, blue tips represent lunged, stream-dwelling tadpoles, red tips represent lungless, pond-dwelling tadpoles and maroon tips represent lungless, stream-dwelling tadpoles. Encircled numbers reference each independent evolution of larval lunglessness in frogs

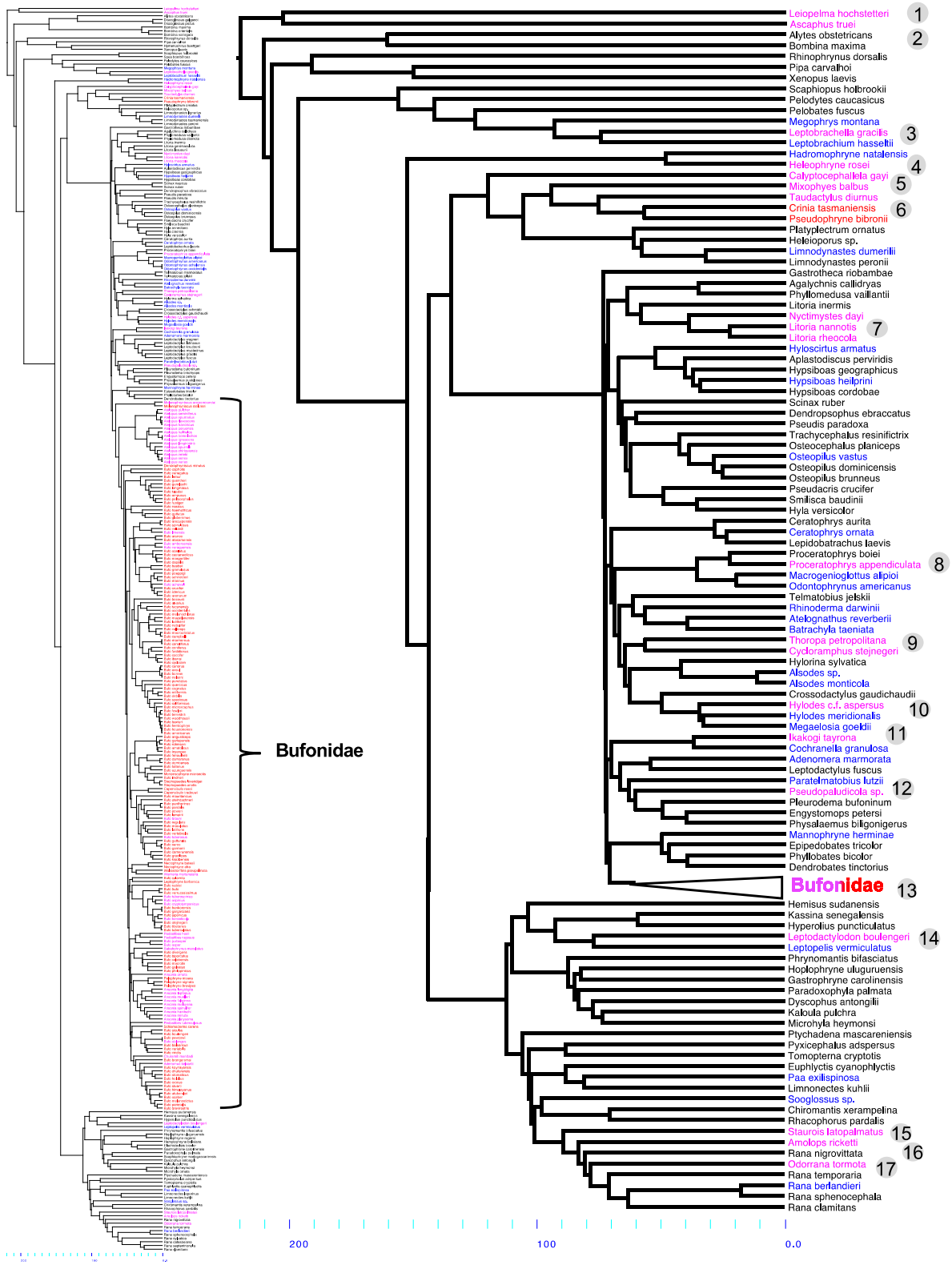
Leiopelma hochstetteri
Ascaphus truei
Alytes obstetricans
Bombina maxima
Rhinophrynus dorsalis
Pipa carvalhoi
Xenopus laevis
Scaphiopus holbrookii
Pelodytes caucasicus
Pelobates fuscus
Megophrys montana
Leptobrachella gracilis
Leptobrachium hasseltii
Hadromophryne natalensis
Heleophryne rosei
Calyptocephallela gayi
Mixophyes balbus
Taudactylus diurnus
Crinia tasmaniensis
Pseudophryne bibronii
Platyplectrum ornatus
Heleioporus sp.
Limnodynastes dumerilii
Limnodynastes peronii
Gastrotheca riobambae
Agalychnis callidryas
Phyllomedusa vaillantii
Litoria inermis
Nyctimystes dayi
Litoria nannotis
Litoria rheocola
Hyloscirtus armatus
Aplastodiscus perviridis
Hypsiboas geographicus
Hypsiboas heilprini
Hypsiboas cordobae
Scinax ruber
Dendropsophus ebraccatus
Pseudis paradoxa
Trachycephalus resinifictrix
Osteocephalus planiceps
Osteopilus vastus
Osteopilus dominicensis
Osteopilus brunneus
Pseudacris crucifer
Smilisca baudinii
Hyla versicolor
Ceratophrys aurita
Ceratophrys ornata
Lepidobatrachus laevis
Proceratophrys boiei
Proceratophrys appendiculata
Macrogenioglottus alipioi
Odontophrynus americanus
Telmatobius jelskii
Rhinoderma darwinii
Atelognathus reverberii
Batrachyla taeniata
Thoropa petropolitana
Cycloramphus stejnegeri
Hylorina sylvatica
Alsodes sp.
Alsodes monticola
Crossodactylus gaudichaudii
Hylodes c.f. aspersus
Hylodes meridionalis
Megaelosia goeldii
Ikakogi tayrona
Cochranella granulosa
Adenomera marmorata
Leptodactylus fuscus
Paratelmatobius lutzii
Pseudopaludicola sp.
Pleurodema bufoninum
Engystomops petersi
Physalaemus biligonigerus
Mannophryne herminae
Epipedobates tricolor
Phyllobates bicolor
Dendrobates tinctorius
Bufonidae
Hemisus sudanensis
Kassina senegalensis
Hyperolius puncticulatus
Leptodactylodon boulengeri
Leptopelis vermiculatus
Phrynomantis bifasciatus
Hoplophryne uluguruensis
Gastrophryne carolinensis
Paradoxophyla palmata
Dyscophus antongilii
Kaloula pulchra
Microhyla heymonsi
Ptychadena mascareniensis
Pyxicephalus adspersus
Tomopterna cryptotis
Euphlyctis cyanophlyctis
Paa exilispinosa
Limnonectes kuhlii
Sooglossus sp.
Chiromantis xerampelina
Rhacophorus pardalis
Staurois latopalmatus
Amolops ricketti
Rana nigrovittata
Odorrana tormota
Rana temporaria
Rana berlandieri
Rana sphenocephala
Rana clamitans
Bufonidae
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
200
100
0.0

Figure 2.2: Rate matrices produced under the dependent model of evolution. Starting states are on the left and final states are on the rate. Rates of changing both characters simultaneously are set to 0, as well as the rates of regaining lungs once lost (see text). In the first matrix, P refers to “pond” i.e. lentic larval life history and S refers to “stream” i.e. lotic larval life history. When used in parentheses, (e.g. lose lungs (P)), this means the rate of losing lungs in a pond-breeding species.

	0,0	0,1	1,0	1,1
	pond, lungless	**pond, lunged**	**stream, lungless**	**stream, lunged**

	0,0	0,1	1,0	1,1
0,0	-	gain lungs (P)	P⇨S (no lungs)	-
0,1	lose lungs (P)	-	-	P⇨S (w/ lungs)
1,0	S⇨P (no lungs)	-	-	gain lungs (S)
1,1	-	S⇨P (w/lungs)	lose lungs (S)	-

Tree 1	0,0	0,1	1,0	1,1
0,0	-	0	16.249	-
0,1	0.408	-	-	6.066
1,0	9.911	-	-	0
1,1	-	16.992	9.133	-

Tree 2	0,0	0,1	1,0	1,1
0,0	-	0	5.817	-
0,1	0.407	-	-	6.068
1,0	2.008	-	-	0
1,1	-	14.763	9.878	-